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Full Name of Author — Nom complet de l'auteur

JAMES PATERSON SMITH

Date of Birth — Date de naissance

28 - 02 - 46

Country of Birth — Lieu de naissance

U.K.

Permanent Address — Résidence fixe

C/O BILL KLUSHIN,
13324 131 St
EDMONTON ALBERTA

Title of Thesis — Titre de la thèse

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Dr B. Oororke, Dr E. D. Jackson

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A STUDY ON THE CONTROL OF MICROBIAL SPOILAGE OF A GAS PACKAGED BAKERY PRODUCT INTRODUCING THE USE OF RESPONSE SURFACE METHODOLOGY

BY

JAMES PATERSON SMITH

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF

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IN

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(Signed) James P. Smith

PERMANENT ADDRESS:

13324 - 131 Street

Edmonton, Alberta T5L 1N7

Dated August 4th, 1982

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled A STUDY ON THE CONTROL OF MICROBIAL SPOILAGE OF A GAS PACKAGED BAKERY PRODUCT INTRODUCING THE USE OF RESPONSE SURFACE METHODOLOGY submitted by JAMES PATERSON SMITH in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

.....
Supervisor

.....

.....

.....

.....

.....
External Examiner

Date August 4, 1982

TO

LUCY MAE SMITH

Two roads diverged in a wood, and I -
I took one less traveled by,
And that has made all the difference.

Robert Frost

ABSTRACT

English style crumpets, a chemically leavened flour based bakery product with a water activity (*aw*) of 0.98 and pH 7, had a shelf life of approximately 14 days when packaged in a CO_2:N_2 (60:40) gas atmosphere and stored at ambient temperature. However, after 14 days packages were visibly swollen due to additional CO_2 production, the product was discolored and possessed a distinct fruity odor when opened.

Microbiological analyses of the product stored at 25°C for 21 days revealed that at 0 days the spoilage microbiota consisted entirely of facultative anaerobic spore-forming strains of *Bacillus licheniformis*. After 7 days the spoilage pattern changed, with lactic acid bacteria (LAB) accounting for almost 100% of the total plate count (TPC) on APT and MRS (pH 5.5) agar medium. Subsequently, the number of LAB declined slightly in relation to the TPC and *Bacillus licheniformis* strains reappeared.

As the LAB increased in numbers, there was a concomittant increase in CO_2 and lactic acid production and a drop in pH of the product. Metabolic activity continued even after the LAB were in the maximum stationary phase. *Leuconostoc mesenteroides* was subsequently identified as the major CO_2 producing isolate.

Challenge studies with *Clostridium sporogenes* PA3679 indicated a public health hazard could exist if the product was contaminated directly with high inocula of *Clostridium*

botulinum spores (>10^3/g), an extremely unlikely situation. While clostridia were isolated from crumpets (<10 MPN/g), these were identified as typical non-pathogenic contaminants of cereal products. The botulism hazard of the presently produced product, despite the anaerobic environment within the package, is, therefore, remote.

Subsequent studies were directed at extension of shelf life. A technique termed Response Surface Methodology (RSM), involving factorial designs and multiple regression analyses and used extensively in optimization studies in chemical engineering, was employed to determine the levels of environmental factors associated with the product which would control the growth of certain spoilage isolates.

Contour plots of the response data showed that *aw* 0.96-0.986, pH 5-6 and a storage temperature of 20-25°C would inhibit CO_2 production by *Leuconostoc mesenteroides*, whereas *aw* 0.94-0.97, 60-70% CO_2 and a storage temperature of 20-23°C would give a mold-free shelf life of 20-30 days. Subsequent reformulation of the product to *aw* 0.962, using linear programming techniques for cost minimization and packaging the product in an atmosphere of 70% CO_2, gave a shelf life at 20°C almost identical to that predicted from the response data.

The advantages of RSM in this study, in food formulation studies and in the whole area of food microbiology are discussed.

Acknowledgements

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1. INTRODUCTION

The shelf life of many perishable foods, such as meat, eggs, fish, poultry, fruit and vegetables is limited by two important factors: the chemical effect of atmospheric oxygen and the growth of aerobic spoilage organisms. While refrigeration is commonly used to control both of these problems, they are not eliminated entirely. Many foods stored under these conditions continue to spoil through the activities of a psychrotrophic microbiota which is, again, predominantly aerobic. Thus, further control measures are necessary.

One logical approach to such additional control is the provision of anaerobic storage conditions which might be achieved through the replacement of air with inexpensive gases such as CO_2 or N_2. The validity of this procedure was established by Killefer (1930) who showed that pork and lamb kept fresh twice as long in 100% CO_2 at 4-7°C compared to storage in air at the same temperature. Subsequently, similar improvements in keeping quality were noted for beef (Haines, 1933), pork (Callow, 1932) and fish (Coyne, 1932) when stored in CO_2 atmospheres.

Thus, at an early date, its effectiveness in controlling aerobic spoilage and, therefore, its potential for exploitation were readily accepted.

Initial commercial application of CO_2-enriched atmospheres used a Controlled Atmosphere (CA) approach. In CA storage the selected concentration of gases, chiefly CO_2,

is strictly maintained at all times. CA has been used for transportation of chilled beef carcasses from New Zealand and Australia (Lawrie, 1974), and to delay physiological changes such as ripening in fruit and vegetables (Tomkins, 1962).

More recently, attention has turned to Modified Atmosphere (MA) storage of food in small convenient retail or distribution units. In MA storage there is no continuous control of the gas mixture, so changes to the atmosphere composition frequently occur as a result of permeation, leakage, absorption of gases by the product and production of CO_2 by the respiratory activity of the food itself or associated microbiota (Wolfe, 1980). Many applications have been suggested. Thus, MA storage, introducing a wide variety of possible combinations of CO_2 (0-100%), N_2 (0-90%), O_2 (0-100%) and sometimes CO (0.5-1%), has been used successfully to extend the keeping quality of beef (Clark and Lentz, 1969; Clark *et al.*, 1976; Christopher *et al.*, 1979), pork (Christopher *et al.*, 1979; Spahl *et al.*, 1980), fish (Banks *et al.*, 1980; Stier *et al.*, 1981), rice (Ory *et al.*, 1980) and peanuts and pecans (Holaday *et al.*, 1979).

Protection against both aerobic microbiological deterioration and flavor and color defects has been claimed. In addition, some consideration has been given to the control by MA of aerobic spoilage of bakery products where mold growth is often the major factor limiting shelf life (Seiler, 1978; Bogadtke, 1979).

The causes of microbial inhibition

The early experiments clearly established that success in controlling aerobic deterioration was not simply dependent upon the elimination of oxygen; rather there was a definite requirement for CO_2 in the gas atmosphere. Working with selected aerobic bacterial isolates from fish, such as *Achromobacter*, *Flavobacterium*, *Pseudomonas* and *Micrococcus*, Coyne (1932) reported that all species were completely inhibited by 100% CO_2 but apparently grew normally in 100% N_2. Although this illogical observation, disagreeing as it does with the definition of "strict aerobe", may have been due to the absence of experimental precautions to ensure absolute purity of the N_2 or CO_2 source, it did suggest that strict aerobes can grow in very low partial pressures of O_2, a conclusion that has since been confirmed by Enfors and Molin (1980). Therefore, while N_2 contaminated with traces of O_2 was not inhibitory, CO_2 appeared to be so.

Carbon dioxide is, in fact, inhibitory to aerobic microorganisms at much lower concentrations. Coyne (1932) demonstrated complete inhibition of aerobic bacteria in a mixture of CO_2:Air (50:50) and partial, but marked, inhibition by 25% CO_2. Similarly, Ogilvy and Ayres (1951) found that 50% CO_2 was sufficient to prevent aerobic spoilage of frankfurters. More recently, Gill and Tan (1980) studied the effect in more detail by examining the effect of various concentrations of CO_2 on the respiration rates of *Pseudomonas* spp., *Acinetobacter* spp., *Altermonas*

putrefaciens, *Enterobacter* spp. and *Brochothrix thermosphacta* (formerly *Microbacterium thermosphactum*). Their results, summarized in Fig. 1.1, demonstrate at least a 30-40% inhibition of this important metabolic process for the aerobic species at a partial pressure of CO_2 equivalent to approx. 30%. In contrast, they found that these CO_2 concentrations had no effect on the facultative anaerobes *Enterobacter* and *Brochothrix*. *Brochothrix thermosphacta* has been reported to tolerate 75% CO_2 (Roth and Clark, 1975) while the genus *Lactobacillus*, studied by Sutherland *et al.* (1977), can multiply in 100% CO_2. Thus, as supported in applied MA storage studies with perishable foods, 20-30% CO_2 may suppress the growth of aerobic bacteria but does little to control facultative anaerobes or microaerophiles when other growth requirements are satisfactorily met.

Mold growth is similarly inhibited by CO_2. Tomkins (1932) reported that 20-30% CO_2 was sufficient to prevent the growth of most meat-associated fungi, while Skovholt and Bailey (1933) and Seiler (1968) used 50% CO_2 to completely suppress mold spoilage of bread and cakes, respectively. Furthermore, as with bacteria, mold species may vary in their sensitivity to inhibition. For example, *Penicillium*, *Mucor* and *Alternaria* apparently grow well in 10% CO_2 at 1°C while *Aspergillus*, *Rhizopus* and *Cladosporium* species are completely inhibited (Yackel *et al.*, 1971). However, all are prevented from growth at concentrations exceeding 30%. Again, this is not simply due to the lower partial pressures

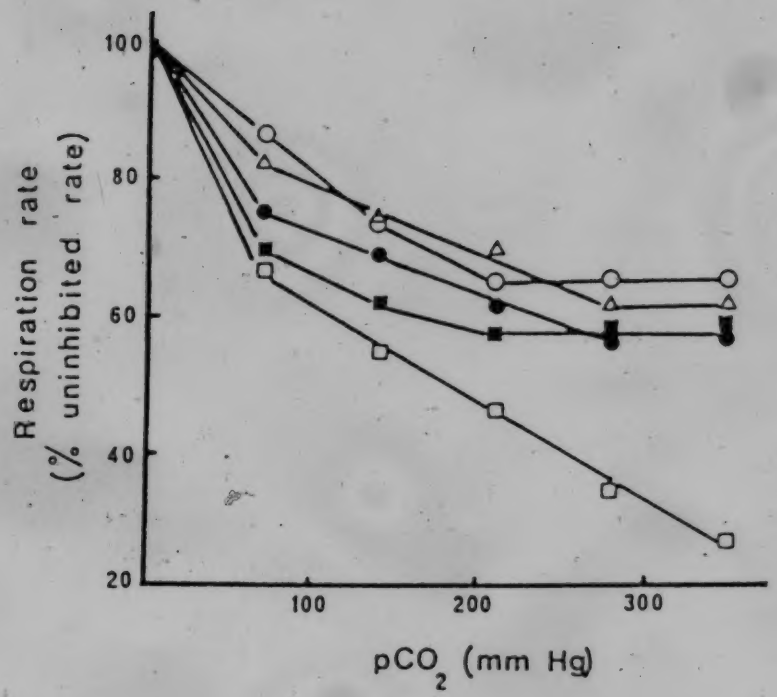

FIG. 1.1 Effect of CO_2 concentration at 30°C on respiration of spoilage bacteria in buffer containing glucose and yeast extracts (Gill and Tan 1980).

○ Nonfluorescent *Pseudomonas*

● Fluorescent *Pseudomonas*

□ *Acinetobacter* sp.

■ *Yersinia enterocolitica*

△ *Aeromonas putrefaciens*

of O_2, since it has been demonstrated that many molds continue to grow normally when the O_2 concentration is maintained as low as 1% (Tabak and Cooke, 1968).

It would appear that, for successful extension of shelf life by MA storage, there is a definite requirement for substantial concentrations of CO_2 in the gas atmosphere, in addition to the total or partial removal of O_2. Under other suitable conditions for growth, this will suppress the growth of aerobic microorganisms but may lead to succession by facultative anaerobes, microaerophiles, or strict anaerobes.

The mechanism of action of CO_2

To date, despite concerted research efforts, the mode of action of CO_2 remains unresolved. Consideration was initially given to the possibility of a pH effect caused by the dissolution of CO_2 into the water phase of the growth substrate (Valley and Rettger, 1927), a theory which continues to be promoted by Enfors and Molin (1981). The latter authors suggest that this phenomenon may explain the enhancement of CO_2 inhibition at low temperatures as a result of increased solubility. However, several writers have shown, when the pH of the medium is lowered by HCl to values equivalent to those achieved under CO_2 atmospheres, bacterial and mold growth is less inhibited (Tomkins, 1932; Haines, 1933). Furthermore, CO_2 will inhibit microbial growth in buffered media (King and Nagel, 1967) and in

naturally buffered foods such as meat (Haines, 1933).

In response to the above observations, Silliker and Wolfe (1980) suggested that the inhibitory effect of CO_2 may be due to intracellular, rather than extracellular, pH changes which could disrupt enzymatic activities. Several investigators have demonstrated this possibility as, *in vitro*, CO_2 has been shown to inhibit the dehydrogenases of *Achromobacter* and *Pseudomonas* species but not of *Proteus* species (Haines, 1933) and the oxalacetate decarboxylase of *Rhizopus nigricans* (Foster and Davis, 1949). Also, as demonstrated by a direct addition of the bicarbonate ion to enzyme preparations from plants, it may have an inhibitory effect on cytochrome oxidase (Miller and Evans, 1956) and succinate dehydrogenase (Bendall *et al.*, 1960).

Other research conflicts with these observations. After establishing a linear relationship between longer generation time and increasing bicarbonate ion for several microbial species under CO_2 atmospheres, King and Nagel (1975) showed that CO_2 did not inhibit extracts of oxalacetate decarboxylase, succinic dehydrogenase or cytochrome oxidase. They observed that CO_2 specifically inhibited malic and isocitric dehydrogenase activities *in vitro* and concluded that the inhibitory effect of CO_2 may be due to its mass action effect on the decarboxylases within the cell.

Other effects at the cellular level, such as disruption of the cell membrane (Sears and Eisenberg, 1961), have been postulated. However, the uncertainty does not detract from

the generally recognized effectiveness of modified atmospheres incorporating CO_2 in the control of aerobic spoilage. Its potential for exploitation, particularly in conjunction with the complimentary effects of other suboptimal environmental factors associated with the food substrates, remains widely accepted.

Factors influencing the inhibitory activity of CO_2

It is evident from the foregoing information that CO_2 concentrations of approximately 50% will stop or severely delay the outgrowth of aerobic microorganisms, regardless of genus, by extending the lag and/or generation time on susceptible foods. However, tolerant microorganisms, although usually in a minority in the biota of perishable foods and thus slower to reach spoilage levels, can still grow. These must be controlled to optimize shelf life.

With regard to further controls, it is widely accepted that microorganisms show greatest tolerance to a single adverse environmental factor when all other conditions are optimal (Troller and Christian, 1980). Conversely, therefore, two or more simultaneous suboptimal environmental conditions will be far more inhibitory than each component considered separately, a synergism which has been amply demonstrated. For example, the reduction in water activity (a_w) required to inhibit the growth of selected facultative or strict anaerobes is much less if nutrients are limiting (Christian, 1955), the temperature is lowered (Liu *et al.*,

1969) or the pH is suboptimal (Baird-Parker and Freame, 1967). Similarly, low pH is far more effective at low temperatures (Emodi and Lechowich, 1969; Ishida *et al.*, 1976). Some studies have also demonstrated complementary inhibitory effects of modified atmospheres and other environmental factors. Thus, Barber and Deibel (1972) demonstrated greater pH tolerance among staphylococci when incubated aerobically as opposed to anaerobically, while Mead (1969) showed an increased requirement of *Clostridium perfringens* for low Eh values when the NaCl content of the growth medium was increased from 0.5% to 5%. Furthermore, modified atmospheres may adversely change the nutritional requirements of some microaerophiles and facultative anaerobes (Banwart, 1979).

Few authors have specifically studied the combined effects of CO_2-enriched atmospheres and complementary factors, despite the early dramatic illustration by Scott (1936) (Fig. 1.2) that relatively minor reductions in a_w improved the inhibitory capacity of CO_2 against aerobic microorganisms; observations that have since been confirmed by Wodzinski and Frazier (1961) and Epstein *et al.* (1970). No similar studies have, apparently, been applied to microaerophiles, facultative anaerobes or strict anaerobes, the possible successors and major spoilage types in gas packaged food products.

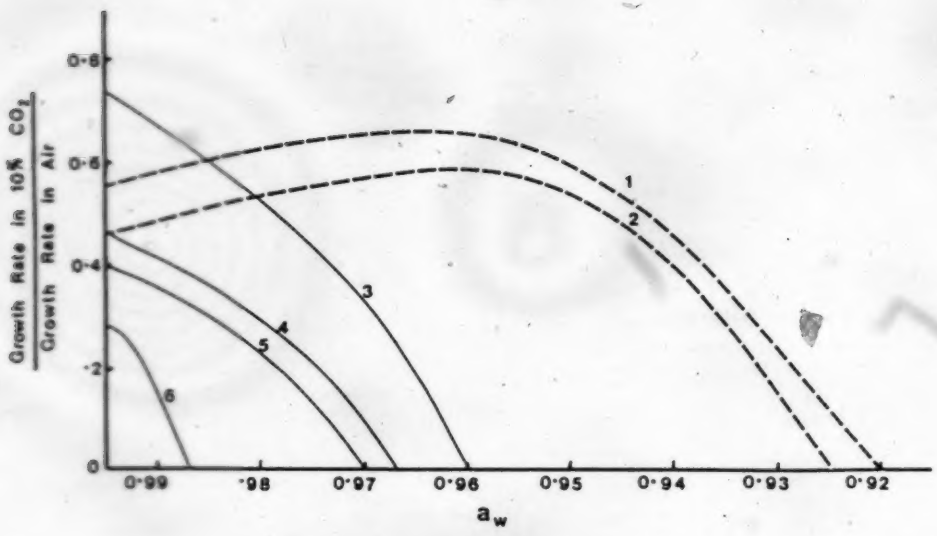

FIG. 1.2 Relation between tolerance to 10% CO_2 and a_w at -1°C (Scott, 1936)

1. *Geotrichoides* Y9
2. Candida Y1
3. Achromobacter No. 5
4. Achromobacter No. 483
5. Achromobacter No. 7
6. *Pseudomonas* sp

While a reduction in storage temperature can provide additional shelf life of gas packaged foods, it is practically the only possible consideration short of irradiation for fresh foods such as meat, fish or vegetables, which cannot be modified or reformulated. Ogilvy and Ayres (1951), Seiler (1968), Clarke and Lentz (1969) and Ory *et al.* (1980) have all reported improved shelf life performance in several foods stored in CO_2-enriched atmospheres at sub-ambient temperatures. Not only may this combination slow the outgrowth of CO_2-resistant contaminants, but it may also increase the inhibitory capacity of CO_2 on susceptible microorganisms (Gill and Tan, 1979) (Fig. 1.3). As mentioned previously, the greater effect of CO_2 at low temperatures has been attributed to increased solubility and associated lower pH values (Enfors and Molin, 1981).

The options for increased spoilage control are more numerous for foods manufactured from several ingredients, such as high moisture bakery products. Here, in addition to low temperatures, consideration may also be given to appropriate formulation changes to manipulate a_w and pH in order to compliment or increase the effectiveness of the CO_2 atmosphere. There is also the real possibility that energy conservation issues could be simultaneously addressed in that the low temperature requirement might be entirely eliminated via this route. Ooraikul (1982) has demonstrated that the retail shelf life of high moisture, English-style

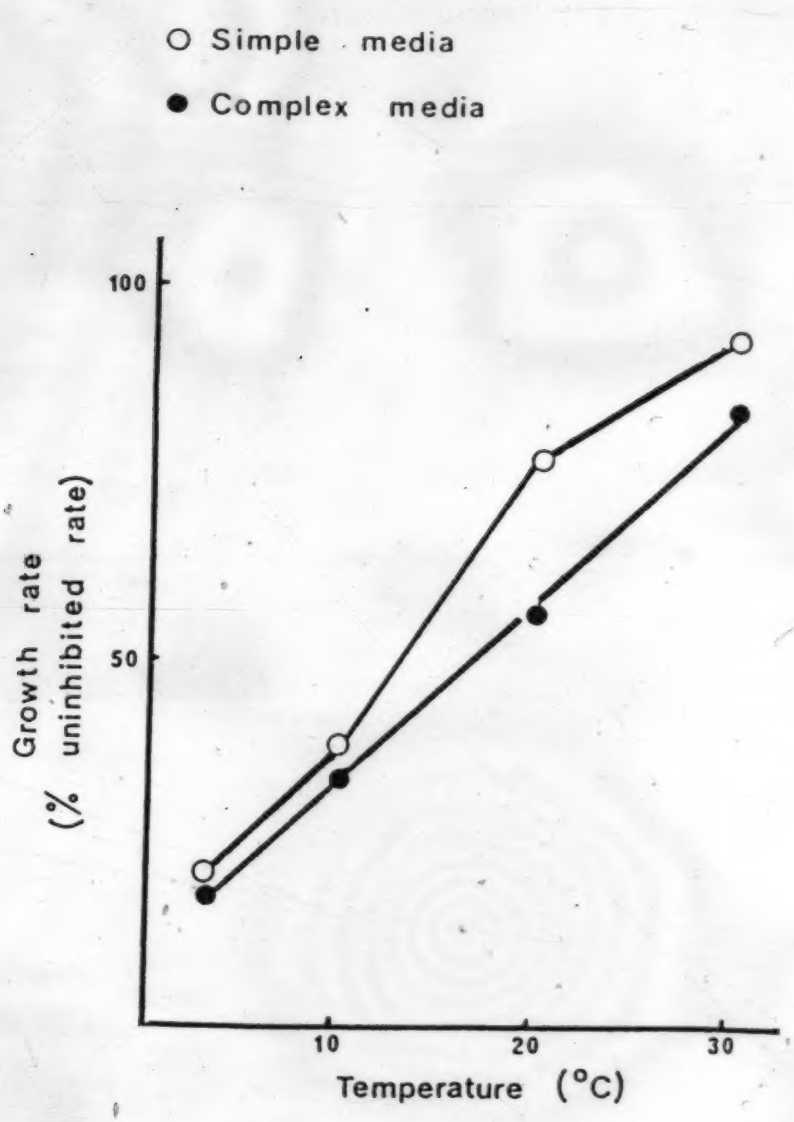

FIG. 1.3. Effect of temperature on the growth rate of *Pseudomonas fluorescens* growing in simple and complex media with CO_2 solution at 150 mm Hg (Gill and Tan, 1979)

crumpets, which have an *aw* of 0.98 and a pH of 7 and are made from flour, water and leavening ingredients, could be increased from 3 to 14 days by packaging in a CO_2/N_2 atmosphere, even at ambient temperatures. Spoilage by mold(s) or yeasts did not occur, but gave way to gas-producing bacteria. Much better product performance might be achieved by appropriate reformulation to slightly reduce the *aw* and pH to further control bacterial successions.

In the light of the above information and discussion, this thesis continues to address the suggested potential for improvement of shelf life of gas packaged crumpets at ambient storage temperature. In addition, it includes attentions to the immediate concerns and expectations of the local manufacturer, Forcrest Foods, Calgary, Alberta. The following objectives were therefore established:

1. Characterisation of the spoilage pattern of the commercial product stored at ambient temperature, with regard to microbial growth, the identity of spoilage species, headspace gas changes, pH and major microbial metabolic byproducts released.

2. Assessment of the potential public health hazard of gas packaged crumpets, since this form of packaging may be conducive to the outgrowth of putrefactive anaerobes such as *Clostridium botulinum*.

3.

Quantification of the range of combinations of selected environmental factors necessary for varying degrees of control of the growth of selected spoilage microorganisms in laboratory model systems.

4.

Reformulation studies on English-style crumpets using linear programming techniques for cost minimization based on information generated from 3.

While objectives 1 and 2 were accomplished in the main using conventional microbiological experimentation, objective 3 became particularly challenging, requiring the simultaneous quantification of the effect of several environmental factors on a growth response. A traditional "one variable at a time" approach, the basis of the most studies in the 2-factor interplay situations, could not provide the necessary detailed description of the ecological dynamics involved. Such approaches assume complete independence of one variable from another and, as such, ignore or at least cannot precisely measure the interactions. A factorial experimental and statistical analysis procedure, termed "Response Surface Methodology" (RSM), was therefore adopted. Although enjoying long term use in the optimization of manufacturing processes in chemical engineering (Box *et al.*, 1978), the techniques are relatively recent in biological studies and almost completely new to Food Microbiology.

An effort has been made to completely describe these techniques in chapter 3 and supporting Appendix II, to demonstrate their benefit to the specific problem at hand and to ultimately provide evidence of their general application to the whole area of shelf life improvement of manufactured foods.

2. STORAGE STUDY ON GAS PACKAGED CRUMPETS

This study was conducted to determine the initial physical, chemical and microbiological characteristics of the commercially produced gas packaged product and to describe changes in these parameters during storage at 25°C.

2.1 Materials and Methods

2.1.1 Sample Collection and Storage

Seventy two random samples were extracted from a day's production lot without regard to production time (10 a.m. or 4 p.m.).

Packages were chilled, packed in cardboard boxes (18 pkg/box) and dispatched to Edmonton by courier for next day's delivery.

Upon receipt, thirty sample packages were selected, coded and subjected to immediate experimentation.

All samples were stored at 25°C and analysed for microbial content, headspace gas metabolites and changes in pH after 0, 3, 7, 14 and 21 days.

2.2 Microbiological Analysis of Gas Packaged Crumpets

2.2.1 Preparation of Samples and Dilutions

Following disinfection of the outside of the packages with ethanol, the film was aseptically cut and 25 g samples removed to include a portion of at least 4 crumpets. An initial 10^{-1} dilution was prepared by adding 225 ml of 0.1% sterile Peptone Water (Difco) and blending on a Colworth 400 Stomacher (Seward & Co., Stanford St., London) for 1 min. Subsequently, a series of decimal dilutions, 10^{-2} to 10^{-4}, was made, again using 0.1% Peptone Water as diluent.

2.2.2 Bacterial Counts

A 0.1 ml amount of the appropriate dilutions was surface plated in duplicate on each of the following media:

APT - For estimation of total numbers

MRS - For selective enumeration of lactic acid bacteria. This media was adjusted to pH 5.6 with 1 N HCl prior to autoclaving to give a final pH of 5.5 ± 0.1.

After inoculation the plates were incubated anaerobically in a Gas Pak Anaerobic jar at 30°C for 48 hr. These conditions were considered superior to aerobic incubation in view of the growth environment within the packages.

2.2.3 Mold and Yeast Counts

Mold and yeast counts were estimated by using a surface plating technique on Potato Dextrose Agar (PDA) containing 10 ppm each of chloramphenicol and chlorotetracycline to suppress bacterial growth.

The required final concentration of antibiotics was achieved by adding 20 ml of a filter-sterilised stock solution (500 mg of each antibiotic/100 ml distilled H_2O) to 980 ml of melted agar precooled to 45°C.

All plates were incubated aerobically at 25°C for 5 days.

All media (APT, MRS and PDA) were prepared from commercially produced dehydrated media (Difco) according to the manufacturer's instructions.

2.2.4 Isolation and Identification of Predominant Spoilage Microbiota

A total of 120 isolates were selected from countable dilutions of APT and MRS agars from two major storage trials. Selection was based on distinctive differences in colony, morphology and upon random selection where colony differentiation was difficult. All isolates were methodically checked for purity and maintained on APT and Nutrient Agar (NA) slants at 4°C.

2.2.5 Identification of Isolates

To expedite identification and redundancy in biochemical tests, isolates were grouped according to the following preliminary tests:

(1) Gram stain and cell morphology

(2) Spore strain (Malachite Green - Safranin)

(3) Catalase test (Slide test using 3% H_2O_2).

Groups were limited to only four combinations of characteristics, namely:

(A) Gram-positive catalase-negative cocci and non-spore-forming rods

(B) Gram-positive catalase-positive spore-forming rods

The above groups were tentatively identified as lactic acid bacteria and *Bacillus* sp. To confirm identification each group was subjected to further biochemical tests.

2.2.5.1 Gram-Positive, Catalase-Negative Cocci and Non-Spore-Forming Rods

(1) API50 *Lactobacillus* Microtube System (Analytical Products, St. Laurent., Quebec)

This simple and economical system incorporates 49 standardized biochemical tests. The API50 trays were inoculated by the procedures outlined in the manufacturer's guidelines, incubated at 30°C and observed for the appropriate reactions after 3, 6, 24 and 48 hr, respectively. Special emphasis was placed on the results of:

Fermentation of carbohydrates

Aesculin hydrolysis

Reduction of nitrate

Production of NH_3 from arginine

Growth in 0.4% Teepol

(2) Additional Selective Tests Outside the Capabilites of the API System

(i) Ability to produce dextran from sucrose

This differential test for certain *Leuconostoc* and *Lactobacillus* species was performed by surface inoculation onto NA containing 10% sucrose (Evans *et al.*, 1956). A known dextran producer *Leuconostoc mesenteroides* (ATCC 27258) was used as control. All plates were incubated at 30°C for 48 hr. The formation of large mucoid colonies indicated dextran production.

(ii) CO_2 production from glucose

The ability to produce CO_2 from glucose distinguishes the homofermentative streptobacteria (e.g. *Lactobacillus plantarium*) and *Pediococcus* species from the heterofermentative betobacteria (e.g. *Lactobacillus viridescens*) and *Leuconostoc* species.

Although the API50 system provides for the test of this characteristic in the glucose fermentation compartment, difficulties were experienced in obtaining complete exclusion of entrapped air during inoculation. It was therefore deemed necessary, in the light of this important characteristic, to determine the heterofermentative activity of isolates in conventional tubes (18 x 150 mm) using a more widely accepted medium, tomato glucose-milk-nutrient agar (Gibson and Abd-el Malek, 1945; Appendix I).

Nine ml of the semisolid medium were liquefied and inoculated with 0.5 ml of bacterial suspension using a heterofermentative strain of *Leuconostoc mesenteroides* (ATCC 27258) as control. Tubes were layered with NA to a depth of 2-3 cm, incubated at 30°C for 14 days in a temperature controlled water bath and examined daily for gas production.

(iii) Growth at 15°C and 45°C

Isolates were inoculated into 9 ml of MRS broth (Difco). One set of tubes was incubated at 15°C (±0.25°C) for 72 hr and another set at 45°C (±0.25°C) in temperature controlled covered water baths (Aquatherm Bath Shaker, Model G86, New Brunswick Scientific Co. Inc., New Jersey). Tubes

were examined visually for growth by comparison against uninoculated controls.

(iv) Growth in 3% and 6.5% NaCl

Test organisms were transferred into 9 ml of MRS broth (Difco) containing 3% and 6.5% NaCl (w/v), respectively, and incubated at 30°C for 48 hr in a temperature controlled waterbath. Tubes were examined visually for growth by comparison against uninoculated controls.

(v) Growth at pH 4.8 and 6.5

Isolates were inoculated into MRS broth (Difco) previously adjusted to pH 4.8 and 6.5 using 1 N HCl and 1 N NaOH. The inoculated media were incubated at 30°C for 72 hr in a temperature controlled water bath and observed for growth against uninoculated control broths.

2.2.5.2 Gram-Positive, Catalase-Positive Spore-Forming Rods

(1) Anaerobic Growth

Isolates were subcultured onto NA plates (Difco), incubated anaerobically in a Gas Pak Anaerobic Jar at 37°C for 48 hr and observed for growth.

(2) Acetoin Production

Isolates were inoculated into 9 ml of MRVP medium (Difco) and incubated at 37°C for 48 hr. The following reagents were used to test for the production of acetoin:

(1) 40% Potassium hydroxide,

(2) α-naphthol (6% in ethanol).

One ml of each reagent was added to the inoculated broths and positive tests (development of red color) recorded after 10 min.

(3) Acid and CO_2 Production from Glucose

Production of acid and gas from glucose was determined in 9 ml of a Peptone Water medium containing 1% (w/v) glucose, 0.0025% (v/v) bromocresol purple as indicator and an inverted Durham tube. Following inoculation the glucose broths were incubated at 37°C for 72 hr. Cultures were observed for acid production, indicated by color change of the indicator to yellow and pockets of gas in the Durham tube.

(4) Reduction in Litmus Milk

Test organisms were inoculated in 9 ml of Litmus Milk (Difco) and incubated at 30°C for 14 days in a temperature controlled water bath. Cultures were examined daily against

an uninoculated control for one or more of the following reactions: acid; acid and clot; proteolysis; gas production.

(5) Hydrolysis of Starch

Petri plates containing NA (Difco) and 0.2% (w/v) soluble starch were surface inoculated and incubated at 37°C for 48 hr. The plates were then flooded with Gram's iodine solution (Difco) and observed for zones of clearing surrounding the colonies.

(6) Decomposition of Casein

Isolates were surface inoculated onto NA plates containing 10% skim milk and incubated at 37°C for 14 days. Plates were then flooded with a solution of the protein denaturing agent, mercuric chloride (Appendix I) and observed for clear zones indicative of casein hydrolysis, surrounding the colonies.

(7) pH Profile

To determine the pH range permitting growth, 0.1 ml amounts of actively growing cultures were transferred to 9 ml amounts of Nutrient Broth (Difco) which had been adjusted over a range of pH values from 4.5-10, in 0.5 pH unit increments, with either 1 N NaOH or 1 N HCl. All tubes were incubated at 37°C for 7 days in a temperature controlled waterbath and examined for growth at regular intervals. Uninoculated tubes at each pH level were used as controls in the event of cloudiness developing from precipitation reactions.

(8) Temperature Gradient Studies

A temperature gradient incubator (Model TGI, Scientific Industries Inc. NY), set within the range 0-70°C, was used to determine the minimum and maximum growth temperatures of presumptive *Bacillus* isolates. Special L-shaped tubes containing 15 ml of Nutrient Broth (Difco) were placed in the holes and allowed to equilibrate overnight. The temperature of each tube was determined with a thermocouple inserted into a duplicate tube. Temperatures were monitored and recorded throughout the duration of the experiment. The pretempered culture tubes were inoculated with 0.1 ml of an actively growing culture of each isolate. Growth was determined by measurement of absorbance (600 nm) at hourly intervals for the first 12 hr and, subsequently, at daily intervals for up to 5 days using a Spectronic 20 spectrophotometer (Bausch and Lomb, Rochester, NY).

2.3 Chemical Analysis

2.3.1 Analysis of Microbial Metabolites by Gas Chromatography

The gas chromatographic (GC) analysis of microbial metabolites released into crumpets during storage was performed to add a further dimension to spoilage analysis with a view to confirming spoilage patterns revealed by conventional methods and therefore test the potential of gas chromatography as a rapid indicator of insipient spoilage and variability.

2.3.2 Sample Extraction

Samples were prepared for extraction by blending 80 g of crumpet and 160 ml distilled water in a Colworth 400 Stomacher for 1 minute. The resultant crumpet slurry was poured into 250 ml polycarbonate bottles and centrifuged in a Sorvall Refrigerated Centrifuge (Model RC2B, Sorvall Inc., Norwalk, Conn.) at 4000 rpm for 10 min. Six ml of the clear supernatant were transferred to a 15 ml tube and acidified with 0.1 ml H_2SO_4 (50% v/v). Four ml were removed for methylation (of nonvolatile acids) according to the procedures outlined below. The acidified aqueous extract remaining was stored at -20°C prior to analysis of volatile acids and alcohols by direct injection.

2.3.2.1 Methylation and Chloroform Extraction For Nonvolatile Acids

Methyl derivatives of nonvolatile acids were prepared by adding 4 ml of boron trifluoride-methanol (125% w/v; Eastman Kodak Co., Rochester, NY) to each 4 ml of acidified aqueous extract and heating the stoppered tubes at 60°C for 30 min in a temperature controlled waterbath. Methyl esters were extracted by addition of 0-5 ml chloroform followed by shaking and centrifugation at 1500 rpm for 10 min in a Sorvall Superspeed Centrifuge (Model SS4, Sorvall Inc., Norwalk, Conn.).

The chloroform layer was removed with a Pasteur pipet to a 12 x 75 mm screw cap culture tube. All prepared samples were stored at -20°C prior to analysis.

2.3.3 Chromatographic Analysis

Gas chromatographic analysis of alcohols, volatile short chain fatty acids (C_1-C_5) and methyl derivatives of nonvolatile metabolites such as pyruvic, lactic, malonic, fumaric and succinic acids was performed using a Varian Aerograph Model 3700 equipped with a Flame Ionization Detector (Varian Associates, Palo Alto, CA). Separation was achieved using a stainless steel column (46 x 0.318 cm) packed with 10% Carbowax 20M on Chromosorb G, AW 80/100 (Chromatographic Specialties, Brockville, Ont.) under the following operating conditions.

Sample size	10 µl
Injector port temperature	170°C
Detector oven temperature	170°C
Carrier gas	Helium
Flow rate	50 ml/min
Detector hydrogen flow rate	30 ml/min
Detector air flow rate	300ml/min

The output was integrated on an Autolab 6300 digital integrator (Autolab, Mountainview, CA) and simultaneously recorded on a dual channel recorder (Cole Palmer Instrument Company, Chicago, IL).

2.3.4 Identification and Quantification of Microbial Metabolites

Identification and quantification of microbial metabolites in test samples were obtained by comparison against retention times and peak areas of known standards (Table 2.1) prepared and treated in an identical manner to extraction procedures previously described for samples (Sections 2.3.2 and 2.3.2.1).

Table 2.1 Standard solutions of volatile fatty acids and alcohols and nonvolatile acids used for comparison with gas chromatograph profiles of sample extracts

Standards	Conc. used (p.p.m)
Volatile Fatty Acids and Alcohols	
Ethanol	780
Propanol	300
Butanol	74
Isoamyl alcohol	88
Amyl alcohol	88
Acetic acid	1100
Propionic acid	1000
Butyric acid	970
Isovaleric acid	1300
Nonvolatile Fatty Acids	
Pyruvic acid	440
Lactic acid	450
Malonic acid	520
Fumaric acid	580
Succinic acid	590

2.3.5 Headspace Gas Analysis

2.3.5.1 Gas Chromatograph

Headspace gases (CO_2, O_2 and N_2) were analysed with a Varian Aerograph Chromatograph Model 90P (Varian Associates, Palo Alta, CA). A two column system adapted from Murakami (1959), shown in Fig. 2.1, was used to obtain a complete chromatogram of all components in the gas mixture from a single injection. Details of the stainless steel columns, packing materials (supplied by Supelco Inc., Bellefonte, CA) and operating conditions are summarized in Table 2.2. Helium was used as the carrier gas at a flow rate of 50 ml/min.

2.3.5.2 Sample Preparation and Analysis of Headspace Gases

Samples were prepared for analysis by attaching a small quantity of RTV silicon sealant (Canadian G.E., Toronto, Ont.) to each package. This technique enabled small amounts of headspace gas (1000 µl) to be withdrawn at regular intervals using a gas tight syringe (Hamilton Co., Whittier, CA) without damaging the integrity of the packaging material. Percentage by volume of gases was calculated from standard curves of O_2, CO_2 and N_2 constructed from relative peak areas which had been integrated with an Autolab Digital Integrator.

TABLE 2.2 Operating parameters for analysis by gas chromatography.

Column	Dimensions	Packing Materials	Operating Temp. °C			Function
			Column	Injection Port	Detection Port	
1	46 x 0.318 cm	Carbosieve S60/80	65	125	125	Separation of CO_2
2	90 x 0.318 cm	Molecular sieve 5A60/80	Ambient	125	125	Separation of O_2 and N_2

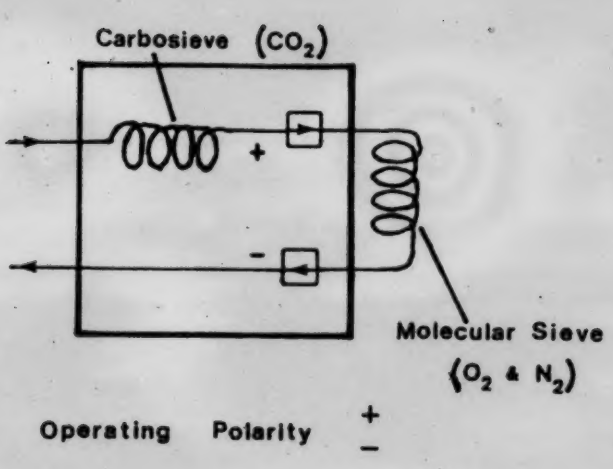

FIG. 2.1 Two column system for headspace gas analysis (Murakami 1959).

2.3.6 pH Measurements of Crumpets

pH measurements were made by immersing the electrodes of a previously calibrated Fisher pH meter (Fisher Scientific Co., Pittsburgh, Penn.) into a 1:2 slurry of crumpet in deionized glass distilled water.

2.4 Results and Discussion

The results of the microbiological and chemical analysis of gas packaged crumpets stored at 25°C are shown in Fig. 2.2 and Table 2.3. The points on each curve represent the mean of 4 packages sampled in duplicate. It is evident from Fig. 2.2 that crumpets had an initial estimated total plate count (TPC) on APT agar of approximately 10^4 CFU/g, which subsequently increased to 10^8 CFU/g after 21 days storage at 25°C. While no lactic acid bacteria (LAB) were detected initially on MRS agar, a dramatic increase in numbers to 10^4 CFU/g was noted after 3 days storage, while after 7 days the LAB accounted for almost 100% of the total population on both APT and MRS agar. The presence of high numbers of LAB was supported by the substantial quantities of lactic acid released into the product after 7 days (Table 2.3). Subsequently, the counts of LAB reached a plateau and then decreased slightly in relation to the TPC. It is of interest to note that similarities between the initial spoilage pattern and microbial development in gas packaged crumpets with those of refrigerated vacuum packed meats (Kempton and Bobier, 1970).

As anticipated due to good manufacturing practice, the initial level of mold contaminants was low (10^2-10^3 CFU/g) and did not increase throughout storage, confirming the effectiveness of the CO_2:N_2 atmosphere in their control. No yeasts were isolated and therefore could not be implicated as the causative agents of additional CO_2 production within

TABLE 2.3 Storage characteristics of gas packaged crumpets after 0, 3, 7, 14 and 21 days storage at 25°C.

STORAGE CONDITION		pH	HEADSPACE GAS, % VOL.		LACTIC ACID (ppm)
TEMP. °C	TIME (DAYS)		O_2	CO_2	
25	0	7.1	1.7	58	0
	3	7.1	1.0	60	0
	7	7.0	0	60	260
	14	6.7	0	62	1140
	21[a]	6.5	0	76	1150

[a] Packages swollen

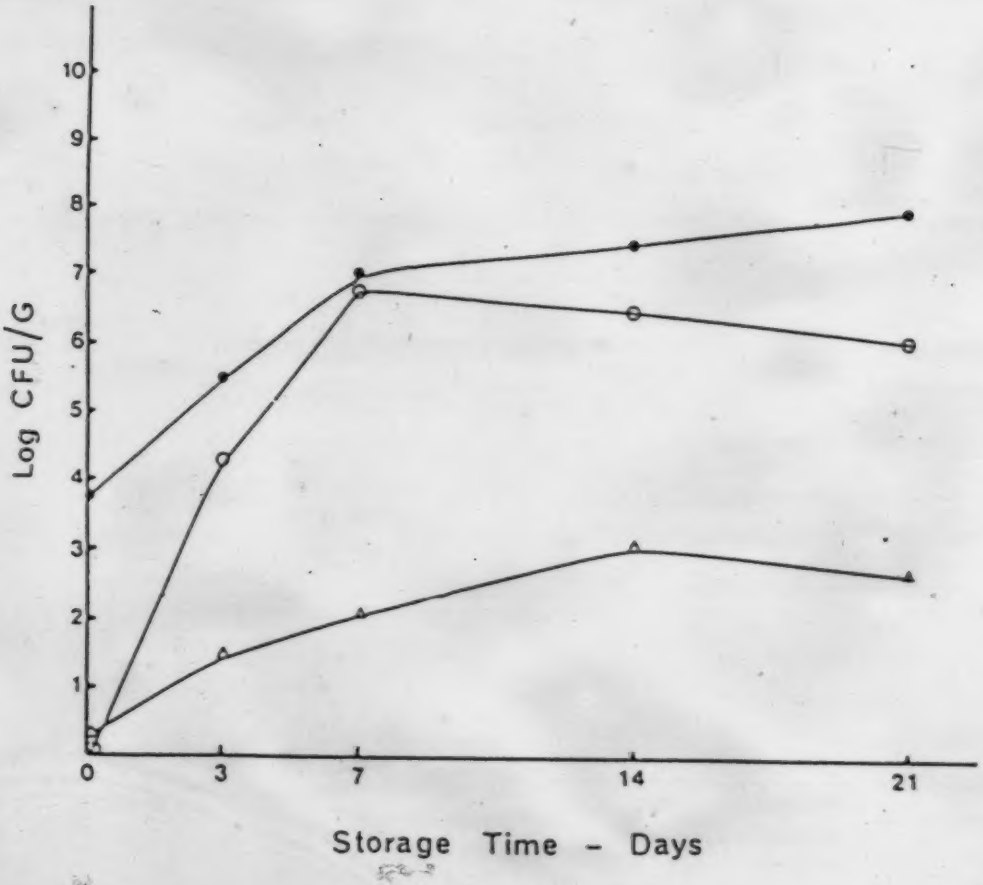

FIG. 2.2 Microbiological analysis of gas packaged crumpets after 0, 3, 7, 14 and 21 days storage at 25°C.

● Total Plate Count *

○ LAB Count *

△ Mold Count /

* Incubated anaerobically

/ Incubated aerobically

the package.

The rapid increase in LAB in relation to the estimated TPC is attributed to a competitive edge over the initial contaminating biota provided by the complete exhausting of residual O_2. This change in the O_2 atmosphere was confirmed by headspace gas analysis (Table 2.3). The subsequent isolation and identification of representative cultures drawn from all points of the growth curves confirmed this hypothesis.

Isolates from APT plates were initially predominantly *Bacillus* sp. with the characteristics detailed in Table 2.4. These characteristics were consistent with *Bacillus licheniformis*, *Bacillus sphaericus* and *Bacillus cereus*, as outlined by Gordon (1973). *Bacillus licheniformis* was the predominant species, accounting for almost 90% of the isolates. These results were not entirely unexpected as this organism is a common contaminant, along with *Bacillus cereus*, of cereal products and is often the causative organism of rope problems in bread.

Isolates on both APT and MRS agar after 7 days were subsequently identified as *Leuconostoc mesenteroides*, *Leuconostoc* or *Pediococcus* sp. and *Lactobacillus* sp., the characteristics of which are shown in Table 2.5.

Although identification of *Leuconostoc mesenteroides* and *Lactobacillus* sp. was fairly routine, some problems were experienced with the identification of Type II isolates. These isolates were morphologically and biochemically

TABLE 2.4 Characteristics of *Bacillus* isolates.

PROPERTY	TYPE I *(B. licheniformis)*	TYPE II *(B. sphaericus)*	TYPE III *(B. cereus)*
Cultural characteristics (APT agar)	Large mucoid creamy warty colony	Small greyish white mucoid colony	Small greyish rhizoid colony
Gram reaction	Gram +ve thin rod	Gram +ve thin rod	Gram +ve thick rod in groups of three
Spores	Round bi-polar spores Sporangium not swollen	Oval sub-terminal spores Sporangium swollen	Oval central spores Sporangium not swollen
Catalase	+	+	+
Anaerobic growth	+	-	+
VP reaction	+	-	+
Acid & gas from glucose	±	-	+
Reaction in litmus milk	Proteolysis	No reaction	Proteolysis
Hydrolysis of starch	+	-	+
Decomposition of casein	+	+	+
pH Range			
Minimum	5.2	5.2	5.2
Maximum	10	10	10
Temperature Range			
Minimum °C	18	12.5	12
Maximum °C	48	47	45

TABLE 2.5 Characteristics of Lactic acid bacteria isolates.

PROPERTY	TYPE I *(Leuconostoc sp.)*	TYPE II *(Leuconostoc or Pediococcus sp.)*	TYPE III *(Lactobacillus sp.)*
Cultural characteristics on	Small greyish white flat colony	Small creamy-white raised colony	Small creamy-white flat colony
Gram reaction and morphology	Gram +ve cocco-bacilli in pairs and chains	Gram +ve cocco-bacilli in pairs and tetrads	Gram +ve slender rods
Catalase	-	-	-
Aesculin hydrolysis	+	+	+
Reduction of nitrate	-	-	-
NH_3 from arginine	-	+	-
Growth in 0.4% Teepol	+	+	+
Dextran synthesis	+	-	-
CO_2 Production	+	-	-
Growth at			
15°C	+	+	+
45°C	-	+	+
Growth in			
3% NaCl	+	+	+
6.5% NaCl	+	+	+
Growth at pH			
4.8 (Initial)	-	+	+
6.5 (Initial)	+	+	+

PROPERTY	TYPE I (*Leuconostoc sp.*)	TYPE II (*Leuconostoc or Pediococcus sp.*)	TYPE III (*Lactobacillus sp.*)
Fermentation of:			
Adonitol	-	-	-
Amydgalin	+	+	+
Arabinose	+	+	+
Cellobiose	+	+	+
Dulcitol	-	-	-
Erythritol	-	-	-
Fructose	+	+	+
Galactose	+	+	+
Glucose	* +	+	+
Inulin	-	-	-
Inositol	-	-	-
Lactose	+	+	+
Maltose	+	+	+
Mannitol	+	+	+
Melizitose	-	-	±
Melibiose	+	+	+
Raffinose	-	-	+
Rhammose	+	+	+
Ribose	+	+	+
Salicin	+	+	+
Sorbitol	-	-	-
Sucrose	+	+	+
Trehalose	+	+	+
Xylose	+	±	-

* CO_2 Production

similar to *Leuconostoc* sp. defined by Garvie (1960) as "Gram-positive catalase-negative cocci producing CO_2 from glucose, acid in litmus milk but failing to produce NH_3 from arginine". However, the Type II isolates failed to produce both NH_3 and CO_2 from glucose. Garvie (1960) also reported the absence of these characteristics in certain strains of *Leuconostoc citrovorum* and *Leuconostoc mesenteroides*, which were subsequently reclassified as Pediococci. According to Garvie (1960), fermentation of carbohydrates is of little assistance in separating the two genera, the results obtained being equally applicable to *Leuconostoc* or *Pediococcus* strains. Our results would concur with the findings of Garvie (1960). Accordingly, Type II isolates were identified as either *Leuconostoc* or *Pediococcus* sp. In contrast to the *Bacillus* isolates, no particular strain was dominant, all colony types being present in equal proportions throughout the storage trial.

Finally, it was shown by the isolation and identification procedures that the shift in population noted towards the end of storage (Fig. 2.2) was due to recurring activity of *Bacillus* sp., in particular *Bacillus licheniformis*. The shift was assumed to be due to nutritional effects, that is the exhaustion of fermentable carbohydrates such as mono- and disaccharides by the LAB, coupled with the ability of *Bacillus* isolates to hydrolyse starch. These species also have the ability to produce lactic acid, as noted in Bergey's Manual of Determinative

Bacteriology (1974) and, therefore, contribute along with LAB to the increased lactic acid found in the product (Table 2.3). Despite the substantial amounts of lactic acid, the pH did not drop to levels likely to be inhibitory to the *Bacillus* sp. and their extracellular amylases.

The most active producer of CO_2 *in vitro* was *Leuconostoc mesenteroides* (Table 2.5). Other species of lactics were not noticeably heterofermentative. Similarly, *Bacillus licheniformis* was inconsistent with regard to this characteristic. Upon fresh isolation from the product, it did produce moderate amounts of gas *in vitro* but tended to lose this ability upon subculturing. This variability in CO_2 production has been confirmed by Gibson and Abd-el-Malek (1945) and is also noted in Bergey's Manual (1974). The other *Bacillus* sp., accounting as they did for only a small proportion of the overall spoilage biota, were not considered to be active contributors to the swelling pattern.

3. ASSESSMENT OF THE BOTULISM HAZARD OF GAS PACKAGED CRUMPETS

A great deal of concern has been expressed recently about the potential public health hazard of food, particularly high protein food such as meat, fish and meat-filled sandwiches packaged under virtually anaerobic conditions. The anaerobic environment which exists initially in such products or is soon created due to the respiratory activity of the product itself or the associated microbiota could be favorable to the outgrowth of and toxin production by *Clostridium botulinum* species.

It is well established that certain vacuum packaged products, when mishandled or in the absence of specific inhibitors such as nitrites, will provide the necessary conditions for the development of and toxin production by *Clostridium botulinum* species (Banwart, 1979). Similar concerns have also been expressed about the use of modified atmospheres, particularly since the trend has been to avoid the addition of microbial inhibitors and, in the case of food such as bakery products, even avoid refrigeration. In some cases these concerns have been confirmed.

Kautter *et al.* (1981) observed that hamburger and sausage sandwiches inoculated with *Clostridium botulinum*, type A, B and E spores, and packaged in an atmosphere of 100% N_2 were toxic after 4-7 days at room temperature, yet appeared perfectly edible. While storage at 12°C or less prevented toxin production by type A and B spores, toxin was

found after 30 days in the gas packaged hamburger sandwiches inoculated with type E spores. However, other workers have not confirmed these concerns. For example, Silliker and Wolfe (1980), in comparing CO_2-enriched atmospheres (up to 60% CO_2) with air packaging, found essentially no difference in the rate of toxin production in pork and chicken inoculated with *Clostridium botulinum* type A and B spores and stored at 27°C. These authors concluded that modified atmospheres did not increase the hazard of botulism. This conclusion has been supported by Stier *et al.* (1981), who found that salmon fillets inoculated with *Clostridium botulinum* type A, B and E spores and stored in air and under modified atmospheres (60% CO_2, 25% O_2, 15% N_2) were toxic within 3 days at 22.2°C regardless of storage atmosphere.

Obviously, the circumstances permitting the growth of and toxin production by *Clostridium botulinum* are complex and not simply dependent on the mere presence of anaerobic conditions. It is widely accepted that other environmental conditions, such as nutritional content, water activity, redox potential, levels of competing organisms and pH, play a major role in determining the ability of individual food products to support the growth of *Clostridium botulinum* strains.

As of yet, there have been no incidents of botulism associated with bakery products. However, toxin production has been reported in canned non-acid bread ($a_w \geq 0.95$) inoculated with *Clostridium botulinum* spores (Waganaar and

Dack, 1954). No similar studies have been conducted with gas packaged bakery products. It would appear from the absence of reported botulism incidents involving gas packaged crumpets during the past 3 years of manufacture and retail in Western and Central Canada that the potential public health risk of this product is minimal or nonexistent. However, controlled laboratory conditions under abuse conditions were necessary to confirm safety.

3.1 Materials and Methods

3.1.1 Preparation of *Clostridium sporogenes* PA3679 Spore Suspension

Clostridium sporogenes PA3679 (ATCC 7955) was grown on PCM agar plates and incubated anaerobically in a Gas Pak Anaerobic jar (BBL) at 25°C for 7 days. This procedure was previously assessed to result in 100% sporulation. Spores were harvested by flooding the plates with a small quantity of 0.1% peptone water, scraping the agar surface with a sterile spatula and centrifuging the resulting suspension in a Sorvall RC-5 refrigerated centrifuge (DuPont Instruments, Connecticut, U.S.A.). Following a single washing and recentrifugation, the spore pellet was resuspended in peptone water and enumerated using an Improved Neubauer Hemacytometer counting chamber (American Optical Scientific Instrument Division, Buffalo, N.Y.). The final concentration

of spores was subsequently adjusted to approximately 50 x 10^3 spores/ml by dilution with 0.1% peptone water. Prior to inoculation the spore suspension was heat shocked at 80°C for 10 min in a temperature controlled water bath.

3.1.2 Preparation of Test Samples

On two separate occasions, one week apart, packages of crumpets were selected at random from the previous day's production and dispatched to Edmonton via courier. Upon receipt, packages were opened as outlined in Section 2.2.1 and the crumpets were aseptically transferred to 30 x 30 cm pouches of 30/60 polyethylene coated nylon film with a total thickness of 90 um (Winpak, Winnipeg, Man.). The film had the following gas transmission rates: O_2 9 cc/m^2/24 hr at 75% relative humidity (RH) and 4°C; CO_2 36 cc/m^2/24 hr at 75% RH and 4°C; water vapor 4 g/m^2/24 hr at 90% RH and 38°C. Samples were gas packaged using Multivac Type AG500 vacuum/gas packaging equipment (W.R. Grace and Co. of Canada, Ajax, Ont.). A vacuum was drawn, the pouch was flushed with the appropriate CO_2:Air mixture and then heat sealed. The proportions of gases from separate cylinders were regulated by a Smith's Proportional Mixer, model. no. 299-006-1 (Tescom, Minneapolis, Minn.).

A total of 20 pouches (1 crumpet per pouch) were prepared and gas packaged by the procedures outlined to provide for two equal batches containing the following gas atmospheres, respectively:

CO_2:N_2 (70:30)

CO_2:Air (70:30)

Each batch was further sub-divided into duplicate lots of 5 for the following treatments:

CO_2:N_2 -- Inoculated

CO_2:N_2 -- Uninoculated Control

CO_2:Air -- Inoculated

CO_2:Air -- Uninoculated Control

Random samples were selected and checked to confirm the desired concentration of gases in the mixture. Gas removal was again performed through a silicon seal attached to the pouch (Section 2.3.5.2). This seal also enabled inoculation of the crumpets without altering the gas atmosphere.

3.1.3 Inoculation and Incubation

One ml of the heat shocked spore suspension (50 x 10^3 spores/ml) was injected through the silicon seal onto the upper surface of each crumpet using a 1 ml disposable syringe. Even distribution was attempted by dropwise application of the suspension and rotation of the crumpet. All samples (test and control) were incubated at 30°C and enumerated after 0, 3, 7, 14 and 21 days, respectively.

3.1.4 Enumeration of Putrefactive Anaerobes

Putrefactive anaerobes were enumerated by a three tube Most Probable Number (MPN) technique adapted from Greenberg *et al.* (1966) using modified Peptone Colloid Medium (PCM)

(Appendix I) and dispensed in 9 ml amounts in screw-capped (16 x 150 mm) tubes. Following inoculation tubes were overlayed with sterilised Vaspar (Appendix I), precooled to 50°C and incubated at 30°C for 7 days. All tubes showing blackening and/or gas production were pasteurised at 80°C for 10 min in a temperature controlled waterbath. A 0.1 ml amount was then transferred to fresh PCM tubes and incubated at 30°C for 5 days. Positive tubes were recorded and the MPN computed according to the method outlined by the ICMSF (1978). Due to the occasional lack of specificity of this test for strictly anaerobic species and the necessity to apply confirmation tests, positive tubes from uninoculated samples were subcultured and maintained on PCM Agar (Appendix I) under anaerobic conditions at 4°C. Random samples were also subjected to headspace gas analysis to ensure packaging integrity had been maintained and that the observed change in putrefactive anaerobe count was a true reflection of the gaseous conditions.

3.1.5 Identification of Putrefactive Anaerobe Isolates from Uninoculated Samples

All isolates from the positive PCM tubes were gram-positive, catalase-negative, spore-forming rods, and were tentatively identified as *Clostridium* species. The following confirmatory tests were carried out:

(1) CO_2 Production from Glucose

CO_2 production was tested in Gibson Abd-el-Malek medium as outlined in Section 2.2.6.1 (ii).

(2) Hydrolysis of Starch

Starch hydrolysis was performed as outlined in Section 2.1.6.2(5), with the exception that inoculated plates were incubated under anaerobic condition in a Gas Pak Anaerobic Jar.

(3) Lecithinase Production

Lecithinase production was determined on NA plates (Difco) supplemented with 10% Bacto Egg Yolk Enrichment 50% (Difco, supplementary literature).

Isolates and a control species, *Clostridium sporogenes* PA3679 (ATCC 7955) were surface inoculated and incubated anaerobically in a Gas Pak Anaerobic Jar at 37°C for 48 hr. Cultures were observed for zones of opalescence indicative of lecithinase activity and for colony characteristics comparable to the control.

(4) Reduction of Nitrate

Isolates were inoculated into 9 ml amounts of Nitrate Peptone Water (1%) broth containing 0.02% potassium nitrate and incubated at 37°C for 48 hr. The following reagents were used to test for the presence of nitrate:

(1) 8.0 g sulphanilic acid dissolved in 1 l of 5 N acetic acid

(2) 5.0 g of α-naphthylamine dissolved in 1 l of 5 N acetic acid

Immediately before use, solutions (1) and (2) were mixed in equal volumes and 0.1 ml of the test reagent added to each culture. A red color, developing in a few minutes, indicated the presence of nitrite and hence the ability of the isolate to reduce nitrate.

(5) Liquefaction of Gelatin

The ability to liquefy gelatin was tested in 9 ml amounts of nutrient broth containing 15% gelatin. Following stab inoculation and incubation at 25°C for 14 days in a temperature controlled waterbath, cultures were precooled in ice water and observed for liquefaction against an uninoculated control broth.

(6) Metabolite Analyses and Mouse Toxicity Tests

Metabolite analyses and mouse toxicity tests were carried out by Dr. F. Jackson, Department of Medical Bacteriology, University of Alberta.

For both tests cultures were grown in Peptone Yeast Glucose (PYG) broth and incubated for 3 days at 35°C. Portions of the broths were extracted and tested for metabolites according to the procedures outlined in the Virginia Polytechnic Institute laboratory manual (Holdeman and Moore, 1972), while 0.5 ml of the remaining culture was injected intraperitoneally into laboratory white mice. The mice were held for 4 days and examined daily for symptoms and death characteristics of *Clostridium botulinum* intoxication.

3.2 Results and Discussion

It is evident from Fig. 3.1 that crumpets packaged under modified atmospheres and challenged with high inoculum levels of *Clostridium sporogenes* PA3679 spores (10^3/g) will eventually support growth to levels of 10^8 MPN/g irrespective of the gas atmosphere used. While this level was reached after only 3 days in crumpets packaged under strictly anaerobic conditions (CO_2:N_2), 7 days were required in CO_2:Air (70:30). Thus, an initial O_2 concentration of 3.5% in a CO_2:Air gas mixture was not sufficient to suppress the growth of high inocula of putrefactive anaerobes. This could have been made possible by the rapid consumption of residual O_2 by the normal spoilage biota of *Bacillus* and lactic acid bacteria in conjunction with the assumed ability of the germinated and actively metabolising spore inoculum to create protective micro-environments at low negative redox potentials. The depletion of O_2 was in fact confirmed by headspace gas analyses, which revealed the existence of totally anaerobic conditions after 3 days in the CO_2:Air mixture.

Silliker and Wolfe (1980) and Stier *et al.* (1981) reported that outgrowth of putrefactive anaerobes could even occur in meat and fish packaged in air alone. Rapid O_2 consumption by spoilage biota, metabolizing high spore inocula and the additional presence of Eh-reducing compounds such as sulfhydryl-containing amino acids may again account for these observations.

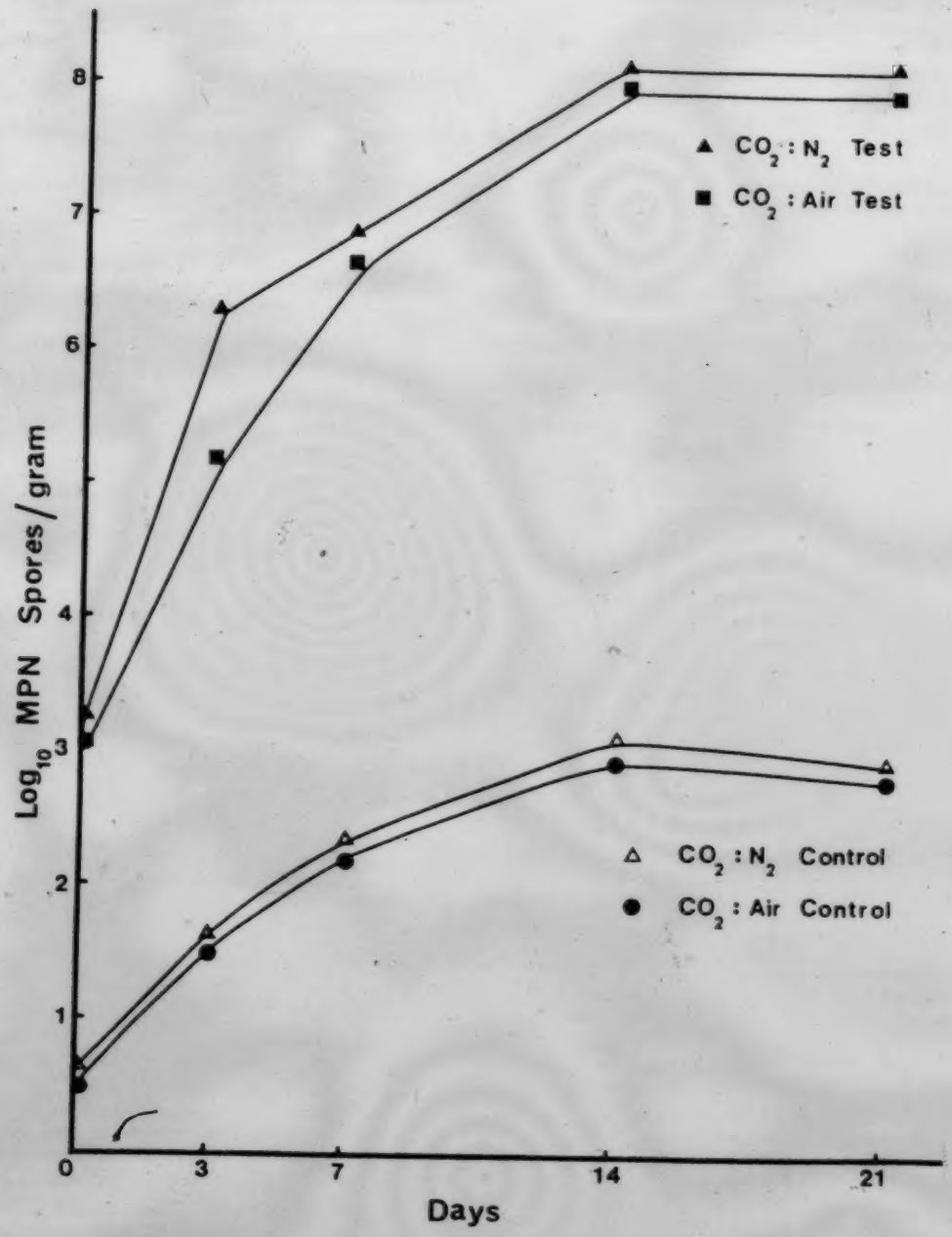

FIG. 3.1 Comparison of the rate of growth of *Clostridium sporogenes* PA3679 in uninoculated and inoculated crumpets packaged under modified atmospheres and stored at 30°C.

It is, therefore, apparent that totally anaerobic atmospheres in gas packaged crumpets do not necessarily enhance the outgrowth of putrefactive anaerobes and, consequently, the potential of a public health hazard from *Clostridium botulinum* when inocula are high. Danger might even exist in crumpets with up to 30% air in the gas mixture. However, in reality high inoculum levels of putrefactive anaerobe spores in gas packaged crumpets would appear unlikely.

Fig. 3.1 shows that the uninoculated crumpets had a putrefactive anaerobe spore content of <10 MPN/g, which did not reach 10^3 MPN/g after 21 days at ambient temperature, again irrespective of the gas atmosphere. Obviously, these low levels of spores could not compete effectively with the lactic acid bacteria which have been shown to become the predominant spoilage biota during storage at 25°C. Support for this conclusion was provided by Johannsen (1965), who reported that the formation of toxin in vacuum packed products was often lower than those not packed in vacuum. He concluded that the micro-aerophilic environment in vacuum packed products encouraged the growth of lactobacilli which antagonized the growth and toxin formation of *Clostridium botulinum*. Furthermore, extensive analysis on numerous occasions on the commercially produced product and, on a single occasion, the flour ingredients, have consistently shown that the putrefactive anaerobe spore content never exceeds 10 MPN/g.

Although a hazardous increase was not observed, it was nevertheless critical to establish their identity and potential to produce toxin. Only one colony type was isolated which possessed the morphological and biochemical characteristics detailed in Table 3.1. Definite similarities with those documented for *Clostridium botulinum* were noted. Production of toxin lethal to mice, a distinguishing characteristic between *Clostridium botulinum* strains and other clostridia, was tested by Dr. F. Jackson (Dept. of Medical Bacteriology, University of Alberta) as outlined in Section 3.1.5. The results shown in Table 3.1 confirm that the isolated PA are not *Clostridium botulinum* strains, but probably strains of *Clostridium sporogenes*. This was confirmed by GC patterns of metabolites produced by crumpet isolates and a known culture of *Clostridium sporogenes* PA3679 (ATCC 7955) in PYG broth, again carried out by Dr. F. Jackson. While the serological types A & B of *Clostridium botulinum* have a similar metabolite profile to *Clostridium sporogenes* with respect to fatty acids (C_2-C_4), neither type produces propanol. The presence of this metabolite is therefore distinctive for *Clostridium sporogenes*. None of the metabolites isolated for *Clostridium sporogenes* or associated with the growth of *Clostridium botulinum* were detected in the product throughout the study, providing further evidence that PA did not increase to significant levels in the product, despite the presence of a totally anaerobic environment towards the end of storage. The PA

TABLE 3.1 Characteristics of *Clostridium* isolates

PROPERTY	
Cultural characteristics (PCM agar)	Large greyish-white + rhizoid colonies with mucoid black or white centre
Gram reaction and morphology	Gram +ve short rods
Spores	Oval; Central to sub-terminal spores Sporangium swollen
Catalase	-
CO_2 production	+
Aerobic growth	-
Hydrolysis of starch	+
Lecithinase production	+
Reduction of nitrate	-
Liquefaction of gelatin	+
Pathogenic for laboratory animals	-
Metabolites	
Acids	Acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, caproic
Alcohols	Ethanol, propanol, butanol, pentanol

were no doubt kept in check by the initial and continuing adverse ratio of these organisms in relation to levels of competitors throughout storage.

These results confirm that the *Clostridium botulinum* content of flour and crumpets is below detectable levels. While no widespread surveys have to date been documented to support this conclusion, Ingram and Handford (1957) did report that the *Clostridium botulinum* spore content of flour and bread dough would be unlikely to exceed 1/g. Kautter *et al.* (1978), working with commercially produced mushrooms packaged under conditions with a headspace O_2 concentration of 1.5%, were unable to detect *Clostridium botulinum* toxin after 7 days. Toxin was, however, found in mushrooms inoculated with 10^3-10^5 spores/g after 3 days under the same incubation conditions. They concluded that the botulism hazard of commercially produced mushrooms was minimal due to the low levels of spores naturally present despite close contact during growth with a substrate likely to harbor *Clostridium botulinum* strains.

It may be concluded that the botulism hazard of gas packaged crumpets is remote, regardless of the storage atmosphere. This conclusion is supported by the absence of botulism incidents over the 3 yr period during which the product has been marketed. Danger would only exist if the product ingredients or the product itself were inadvertently contaminated directly with high levels of *Clostridium botulinum* spores, an extremely unlikely situation and one

which could effectively be guarded against by simple quality control measures.

4. DETERMINATION OF THE SIGNIFICANT GROWTH FACTORS AND THEIR RESPECTIVE LEVELS FOR THE CONTROL OF PREDOMINANT SPOILAGE ISOLATES FROM GAS PACKAGED CRUMPETS .

This study was undertaken in response to the recognition that control of spoilage of this bakery product and, to a large extent, the spoilage of any food product is dependent upon a complete understanding of the environmental factors influencing the growth of spoilage microorganisms. Factors which may influence the growth of the organisms in this product include a_w, pH, storage temperature, level of inhibitor and CO_2:Air ratio.

Having identified the predominant spoilage microbiota, one might consult published data to obtain the limiting values of the above factors for each of the individual species and consider application directly to possible product reformulation and spoilage control. However, the published data have in the main been generated by classical microbiological studies involving one variable at a time. A consequence of this approach is the assumption of complete independence of one variable from another and, therefore, no interaction among variables. As a result, the level of each parameter necessary for control has likely been exaggerated.

Adequate quantitative description of the effect of environmental factors, to include important interactions among variables on microbial growth, can only be achieved by their simultaneous evaluation. One such technique which can be used to investigate such problems is Response Surface

Methodology (RSM), involving factorial experimental designs and multiple regression statistical analyses and a procedure which has been adopted successfully in chemical engineering and food formulation studies (Box *et al.*, 1978; Henika, 1982).

RSM techniques were applied in model laboratory systems in this study to:

(a) select the significant environmental factors from those parameters that are considered important, within the constraints of this particular bakery product, and are necessary to control the growth of chosen spoilage organisms; and

(b) measure the levels of significant factors with due consideration to all possible interactions for varying degrees of control of these microorganisms.

For the convenience of the reader, the essence of the procedure for RSM has beed distilled from the available texts on the subject and presented in Appendix II. To avoid duplication in Materials and Methods, and Results and Discussion, the reader should refer to the pertinent section of Appendix II where appropriate.

4.1 Materials and Methods

4.1.1 Selection of Test Organisms

4.1.1.1 *Leuconostoc mesenteroides*

This organism was selected for further study as it had been demonstrated previously to be the most active CO_2 producer *in vitro* and, hence, to contribute significantly to volume changes in the gas packaged product. An adequate understanding of the significant factors and levels of each factor required to control growth was therefore necessary to optimize the shelf life of the product.

4.1.1.2 *Aspergillus niger*

Although *Penicillum* sp. were the predominant contaminants of the gas packaged crumpets at the time of sampling, this may not represent a consistent situation. Mold contamination of bakery products is frequently subjected to seasonal variation. It was therefore decided to choose a common spoilage mold of a wide range of bakery products which tends to be among the most resistant to adverse environmental factors, particularly with regard to *aw*. Factors controlling this species would therefore likely control all other mold contaminants with the exception of highly xerophilic but uncommon and difficult to handle species such as *Eurotium amstelodami*.

4.1.2 Selection of Factors Under Study

The factors under study, namely *aw*, pH, storage temperature, CO_2:Air ratio, concentration of inhibitor and inoculum levels, were selected; (1) from those recognized as influencing microbial growth and (2) on the basis of the commercial product as it now exists and the practical possibilities for reformulation.

4.1.2.1 Water Activity

Water activity (*aw*) is regarded as one of the most important environmental factors used to control microbial growth. The range of *aw* values used in the *Leuconostoc mesenteroides* study (0.94-0.98) was selected on the basis of *aw* constraints for the possible reformulation of the product. In view of the tolerance of molds to a wider range of *aw* values in comparison to bacteria, an initial range of *aw* values form 0.85-0.95 was selected for the *Aspergillus niger* screening study.

4.1.2.2 pH

Levels of pH chosen (5-7) were selected on the basis of the acidophilic nature of both molds and lactic acid bacteria and the pH of the presently formulated product.

4.1.2.3 Storage Temperature

This is an obvious variable, but had to be restricted to the range of temperatures (20-25°C) most likely encountered during distribution and in the retail

environment.

4.1.2.4 CO_2:Air Ratio

Although the product is currently packaged in a CO_2:N_2 atmosphere, the added N_2, as discussed in the general introduction, has not been demonstrated to have any antimicrobial effect and may, therefore, represent an unnecessary extra production cost. Two CO_2:Air ratios (0:100; 70:30) were studied.

4.1.2.5 Inhibitor

In this study the use of microbial inhibitors was limited to potassium sorbate on the basis of its ease of solubility at room temperature and its incorporation in the presently produced product. Although used mainly as an antimycotic agent, it has been shown to be effective against catalase positive bacteria and also to inhibit the growth and toxin production of catalase negative strains of *Clostridium botulinum* (Banwart, 1979). Upper levels in this study were restricted to the maximum amounts legally permitted in bakery products (1300 ppm).

4.1.2.6 Inoculum Level

Inoculum level was considered important from the viewpoint that relatively high cell numbers can tolerate or adapt quicker to adverse environmental conditions than low numbers. Levels of *Leuconostoc mesenteroides* and *Aspergillus niger* were selected on the basis of the range of levels

(10^2-10^4 CFU/g) which might occur in the product under good manufacturing conditions.

4.1.3 Factorial Experimental Designs

The factorial experimental designs and associated experimental runs included in RSM for *Leuconostoc mesenteroides* and *Aspergillus niger* are shown in the following series of Tables. The reader is referred to the specific experimental design for details of levels of each of the above factors.

(a) *Leuconostoc mesenteroides*.

Table 4.1. A preliminary 2^{4-3} fractional factorial screening design to determine the environmental factors influencing growth.

Table 4.2. A 2^{4-3} fractional factorial screening design with *aw* held constant. A preliminary screening design with an *aw* range of 0.94-0.98 indicated that *aw* below 0.96 masked the effects of all other factors. In this modified design *aw* was held constant at 0.98 in all experimental runs. Runs 2, 4, 6 and 8 of the preliminary screening design composed Block 1 and these were supplemented with 4 additional runs which composed Block -1.

Table 4.3. Full factorial design for estimating the first order polynomial model.

Table 4.4. Additional experimental trials determined from path of steepest ascent method.

Table 4.5. Uncoded levels of central composite design (CCD)

for estimating the second order polynomial model.

(b) *Aspergillus niger*.

The set of factorial experiments for *Aspergillus niger* was identical in principle to those for *Leuconostoc mesenteroides*, but differed in the levels of certain factors employed. The details of experimental runs are shown in Tables 4.10-4.15.

4.1.4 Measured Growth Responses

For all stages of factorial studies involving *Aspergillus niger* "days to visible growth" was chosen as the appropriate measure to determine the influence of environmental factors on growth. A similar response was chosen for *Leuconostoc mesenteroides* in the initial screening design. However, in later designs the measured response for this organism was changed to CO_2 production.

4.2 Materials and Methods

4.2.1 Media Used

MRS agar (Difco) was used as the basal medium for *Leuconostoc mesenteroides* in the initial factorial screening designs, while MRS broth (Difco) was used in all subsequent studies.

For *Aspergillus niger* PDA was used as the basal medium.

Following adjustments of the media, with the exception of pH, which was done after sterilisation, all agar media were autoclaved at 121°C for 15 min and dispensed in 50 ml quantities in 150x15 cm Petri plates (Honour Plastics Ltd., Toronto). In the case of MRS broth, subsequently used for the *Leuconostoc mesenteroides* study, 9.9 ml amounts were aseptically dispensed into sterile 25 ml measuring cylinders. Both the MRS broth and cylinders were sterilised by autoclaving at 121°C for 15 min.

4.2.2 Addition of Potassium Sorbate

Amounts of 0.5 ml and 1 ml of a 13% (w/v) stock solution of potassium sorbate were added to each 100 ml of media to give final concentrations (w/v) of 0.065% and 0.13%, respectively.

4.2.3 Adjustment of *aw*

All media were adjusted to the *aw* values shown in the respective designs using combinations of NaCl and dextrose. The molal concentration required in each case was obtained from the data of Christian (1980). Prior to autoclaving, a vacuum was drawn on each sample to remove noncondensable gases. This was done in order that the *aw* value could be accurately confirmed by the manometric technique of Taylor (1961), as modified by Lewicki *et al.* (1978). The instrumentation is shown in Plate 4.1.

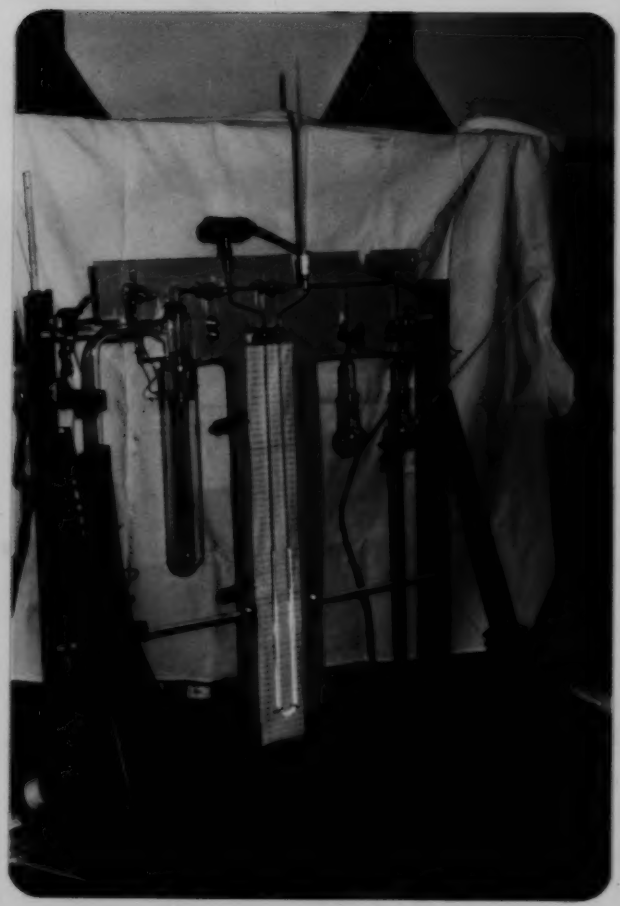

Plate 4.1 Apparatus to measure a_w manometrically

Random checks on the accuracy of this technique were made using saturated solutions of inorganic salts with *aw* values ranging from 0.75-0.96.

All *aw* measurements for both saturated salt solutions and adjusted media were within ±0.2% of the expected *aw* value.

4.2.4 Adjustment of pH

The pH's of all media were adjusted after autoclaving using 1 N HCl or 1 N NaoH. pH measurements were made with a calibrated Fisher pH meter (Fisher Scientific Co., Pittsburgh, PA).

4.2.5 Preparation of Inoculum

4.2.5.1 *Leuconostoc mesenteroides*

An active culture of *Leuconostoc mesenteroides* was prepared in APT broth from a previously isolated and identified strain maintained on APT slopes at 4°C. Optimum activity was assured by passaging 0.1 ml quantities through a sequence of fresh APT broths incubated at 30°C for 18-24 hr. A final concentration, as determined by a surface plate count on APT agar, of 30 x 10⁹ cells/ml was achieved.

4.2.5.2 *Aspergillus niger*

Aspergillus niger (ATCC 11414) spores were produced on PDA slopes incubated at 25°C. Spores were washed off the slopes with 0.1% Peptone Water and decanted into sterile

tubes. This suspension was enumerated using an Improved Nebauer Haematocytometer Counting Chamber and appropriate dilutions were made to give stock solutions containing 20,000 and 2,000 spores/ml. After dilution, the stock solutions were again enumerated to confirm the desired spore concentration. At this time clumping of spores was not observed, thus negating the requirement for addition of surfactant.

Checks for spore viability were periodically made on PDA under aerobic incubation conditions at 25°C.

4.2.5.3 Inoculation

In screening designs which required specific levels of the respective organisms, 0.1 ml of the appropriate dilution was surface plated onto MRS and PDA agar plates. All plates were dried for 30 min and then gas packaged as outlined in the next section. The inoculation of test media used in subsequent designs was held to one level, i.e. 0.1 ml/plate for *Aspergillus niger* and 0.1 ml/graduated cylinder for *Leuconostoc mesenteroides* from the appropriate stock solution.

4.2.6 Incubation Conditions

4.2.6.1 (a) Gas Atmospheres

All inoculated plates were packaged in a 30 x 30 cm pouch (1 plate/pouch) of the 30/60 polyethylene-coated nylon film previously described in section 3.1.2. Random checks

were made on the gas packaged plates using GC techniques outlined in Section 2.3.5.2 to ensure that the appropriate CO_2:Air ratio had been achieved.

4.2.6.2 (b) Storage Temperature

All inoculated and packaged media were stored at the temperature conditions dictated by the respective designs.

For storage temperatures of 25 and 30°C, a Fisher Isotemp (±0.25°C) incubator (Fisher Scientific Co., Pittsburgh, PA) was used, while for media stored at 20°C a Psycrotherm incubator shaker, model C26 (±0.1°C) was used (New Brunswick Scientific Co. Inc., New Brunswick, NJ).

4.2.7 Statistical Analysis

The regressions were computed using the University of Alberta APL Statistical Package (Smillie, 1969), while the eigenvalue vectors were computed using an APL eigenvalue program of Computing Services, University of Alberta. Contour plots were drawn using the Interactive Statistical Graphics Package of Computing Services, University of Alberta.

4.3 Results and Discussion

4.3.1 (a) *Leuconostoc mesenteroides*

Regression analysis of the preliminary 2^{4-1} factorial screening design (Table 4.1) and examination of the fitted coefficients using Student's two-tailed t-test (1 d.f.) revealed that *aw* was the only significant factor influencing the growth of *Leuconostoc mesenteroides*. Further review of the literature indicated that the lack of significance of the other factors was most likely due to the range of *aw* values selected (0.94-0.98), which masked the effect of all other variables. Consequently, a further 2^{4-1} design (Table 4.2), with *aw* held constant at 0.98, was carried out to determine the effect of the other factors on growth. The regression equation obtained from analysis of the data was:

$$y = \beta_0 + 0X_1 - 0.75X_2 + 0X_3 + 0X_4 + 0X_5 - 0.1X_6$$

Only pH and temperature were significant, while CO_2:Air ratio, concentration of inhibitor and inoculum levels were not significant. The nonsignificant factors were, therefore, not used in subsequent factorial designs. The results of inoculum levels were somewhat unexpected in that high inoculum levels are usually more adaptable to adverse environmental conditions than low levels. However, the effect of CO_2 and potassium sorbate on growth was not unexpected. Potassium sorbate, although used mainly as an antimycotic agent, has been shown to be effective against catalase positive bacteria but not catalase negative

TABLE 4.1 A preliminary 2^{6-3} factorial screening design to determine factors influencing the growth of *Leuconostoc mesenteroides*.

a_w	pH	INOCULUM LEVEL (CELLS/ml)	CONC. OF INHIBITOR (%)	CO_2:AIR RATIO(%)	STORAGE TEMP(°C)	DAYS TO VISIBLE GROWTH
0.94	5.25	10^2	0.13	50	20	> 4
0.98	5.25	10^2	0.065	0	30	2.5
0.94	7.25	10^2	0.065	50	30	> 4
0.98	7.25	10^2	0.13	0	20	2
0.94	5.25	10^4	0.13	0	30	> 4
0.98	5.25	10^4	0.065	50	20	3.5
0.94	7.25	10^4	0.065	0	20	> 4
0.98	7.25	10^4	0.13	50	30	1

VARIABLES	LEVELS -1	0	+1
a_w (X_1)	0.94	0.96	0.98
pH (X_2)	5	6	7
Inoculum (cells/ml) (X_3)	10^2	10^3	10^4
Conc. of inhibitor (%) (X_4)	0.065	0.0975	0.13
CO_2:Air Ratio (%) (X_5)	0:100	25:75	50:50
Storage Temp. (°C) (X_6)	20	25	30

TABLE 4.2 A 2^{6-3} factorial screening design with a_w held constant at 0.98 to determine factors influencing the growth of *Leuconostoc mesenteroides*.

BLOCKS	pH	INOCULUM LEVEL CELLS/PLATE	CONC. OF INHIBITOR (%)	CO_2:AIR RATIO(%)	TEMP(°C)	DAYS TO VISIBLE GROWTH
1	5.25	10^2	0.065	0	30	2.5
1	7.25	10^2	0.13	0	20	2
1	5.25	10^4	0.065	50	20	3.5
1	7.25	10^4	0.13	50	30	1
-1	5.25	10^2	0.13	50	20	3.5
-1	7.25	10^2	0.065	50	30	1
-1	5.25	10^4	0.13	0	30	2.5
-1	7.25	10^4	0.065	0	20	2

organisms such as *Leuconostoc mesenteroides*. Furthermore, lactic acid bacteria have been show to tolerate and even grow in the presence of high concentrations of CO_2 (>70%) (Sutherland *et al.*, 1977).

The three most important factors influencing growth, namely *a*w, pH and storage temperature, were further studied to determine their effect on CO_2 production. Two combined first order designs ($2x(2^3+4)$ were carried out (Table 4.3). The regression equation obtained from analysis of results after 72 hr was:

$$y = \beta_0 + 218.75X_1 + 4.35X_2 + 0.26X_3$$

All estimated β values remained significant at the 0.05 level (19 d.f.). The positive nature of the regression coefficients indicated that CO_2 production would increase if the levels of each factor increased. This was tested using the path of steepest ascent shown in Table 4.4. The predicted levels of CO_2 production were obtained by multiplying the values of *a*w, pH and temperature in each run by the linear regression coefficients for each factor. Examination of the experimental runs (Table 4.4) shows that CO_2 production did increase in a similar manner to that predicted.

On the basis of these results, another first order model would normally be performed to determine the additional levels of factors required to maximize CO_2 production. This would have entailed using unrealistic base levels of *a*w 0.99-1.0, an unrealistic range of *a*w values.

TABLE 4.3 A $(2x(2^3+4)$ design for development of first order polynomial model

BLOCKS	LEVELS			CO_2 PRODUCTION (ml)	
	a_w	pH	Temp (°C)	48 hrs	72 hrs
-1	0.97	5.75	20	1	3.5
-1	0.99	5.75	20	7	9.3
-1	0.97	7.25	20	5	8.6
-1	0.99	7.25	20	7.5	9.7
-1	0.97	5.75	30	1	4.5
-1	0.99	5.75	30	10.9	12
-1	0.97	7.25	30	8.8	11.4
-1	0.99	7.25	30	11.7	12.7
-1	0.98	6.5	25	9.8	10.9
-1	0.98	6.5	25	9.8	10.9
-1	0.98	6.5	25	9.5	10.2
-1	0.98	6.5	25	8	11.1
1	0.97	5.25	20	0	0
1	0.99	5.25	20	2.1	5.2
1	0.97	6.75	20	4.8	9
1	0.99	6.75	20	8	10,5
1	0.97	5.25	30	0	0
1	0.99	5.25	30	8.6	11.8
1	0.97	6.75	30	10	11.9
1	0.99	6.75	30	11.2	12.7
1	0.98	6	25	9.7	11.2
1	0.98	6	25	9.7	11.2
1	0.98	6	25	8.9	10.9
1	0.98	6	25	9	11.7

TABLE 4.4 Possible levels derived from path of steepest ascent approach of factors for CO_2 production after 72 hours by *Leuconostoc mesenteroides*

LEVELS			CO_2 PRODUCTION (ml)	
a_w	pH	Temp (°C)	Predicted	Observed
0.97	5.25	25	4.50	0
0.975	5.75	25	6.85	3.7
0.98	6.25	25	9.20	9.8
0.985	6.75	25	11.55	13.5
0.99	7.25	25	13.90	14.8
0.995	7.75	25	16.25	16.2
0.97	5.25	20	3.18	0
0.975	5.75	20	5.53	1.85
0.98	6.25	20	7.87	6.35
0.985	6.75	20	10.22	10.85
0.99	7.25	20	12.57	11.8
0.995	7.75	20	14.92	13.65

Therefore, a 3 factor CCD, the construction of which is outlined in Appendix II, was performed. The levels of a_w, pH and temperature used in each run are shown in Table 4.5. The value of α in this design was obtained from Myers (1976). The predicted responses for CO_2 production after 48 and 72 hr, respectively, were generated from the respective regression coefficients, as outlined earlier for the path of steepest ascent. The second order regression equation obtained from analysis of the coded data (Table 4.6) was:

$$y = \beta_0 + 0.49X_1 + 0.57X_2 - 0.68X_3 - 0.13X_1^2 - 0.31X_2^2 + 0.21X_3^2 - 0.2X_1X_2 - 0.025X_1X_3 - 0.4X_2X_3$$

Examination of the fitted model using Student's t-test indicated that main factors were significant at the 95% level (5 d.f.), but cross-products and quadratic terms were not significant.

The stationary point (X_0) for the fitted surface was obtained by the equation shown in Table A14, Appendix II. The coded and uncoded values of a_w, pH and temperature at the stationary point, which are well within the experimental range, are shown in Table 4.7. The expected yield of CO_2 at the stationary point, y_0, calculated using the equation shown in Table A15 (Appendix II) was 15.1 ml. Examination of the fitted second order equation, expressed as its canonical form, i.e.:

$$y = y_0 + 0.27w_1^2 - 0.09w_2^2 - 0.409w_3^2$$

indicated that two of the eigenvalues were negative and a third positive. The nature of the fitted surface was

TABLE 4.5 A Central Composite Design for k=3 for development of second order polynomial model

LEVELS			CO_2 PRODUCTION (ml)			
			Predicted		Observed	
a_w	pH	Temp(°C)	48 hrs	72 hrs	48 hrs	72 hrs
0.98	7	21	8.09	10.66	9.5	11.5
0.99	7	21	10.37	12.84	10·5	13.5
0.98	8	21	11.13	14.01	12	14.5
0.99	8	21	13.40	16.19	12.5	14.3
0.98	7	24	9.10	11.45	14.5	15
0.99	7	24	11.37	13.64	14.5	15.5
0.98	8	24	12.13	14.80	13.5	15
0.99	8	24	14.41	16.99	15	16.1
0.975	7.5	22.5	8.98	11.63	13	13.5
0.995	7.5	22.5	13.53	16.01	12.5	15.5
0.985	6.7	22.5	8.82	11.14	10.1	13
0.985	8.3	22.5	13.68	16.50	13.5	15
0.985	7.5	20	10.41	13.16	12.8	15
0.985	7.5	25	12.09	14.48	14.7	15.9
0.985	7.5	22.5	11.25	13.82	12.9	14.7

TABLE 4.6 Coded main effects and interactions used to compute second order polynomial model

a_w	pH	Temp	a_w^2	pH^2	$Temp^2$	a_w pH	a_w Temp	pH Temp	CO_2 PRODUCTION (ml) 48 HRS	72 HRS
-1	-1	-1	1	1	1	1	1	1	9.5	11.5
1	-1	-1	1	1	1	-1	-1	1	10.5	13.5
-1	1	-1	1	1	1	-1	1	-1	12	14.5
1	1	-1	1	1	1	1	-1	-1	12.5	14.3
-1	-1	1	1	1	1	1	-1	-1	14.5	15
1	-1	1	1	1	1	-1	1	-1	14.5	15.5
-1	1	1	1	1	1	-1	-1	1	13.5	15
1	1	1	1	1	1	1	1	1	15	16.1
-1.68	0	0	2.82	0	0	0	0	0	13	13.5
1.68	0	0	2.82	0	0	0	0	0	12.5	15.5
0	-1.68	0	0	2.82	0	0	0	0	10.1	13
0	1.68	0	0	2.82	0	0	0	0	13.5	15
0	0	-1.68	0	0	2.82	0	0	0	12.8	15
0	0	1.68	0	0	2.82	0	0	0	14.7	15.9
0	0	0	0	0	0	0	0	0	12.9	14.7

	LEVELS				
VARIABLES	-1.682 (-α)	-1	0	+1	1.682 (α)
a_w (X_1)	0.975	0.98	0.985	0.99	0.995
pH (x_2)	6.7	7	7.5	8	8.3
Temp (°C) (X_3)	20	21	22.5	24	25

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TABLE 4.7 Values of coded and uncoded variables at the stationary point on the fitted surface

Variables	LEVELS	
	Coded	Uncoded
a_w	$X_{1_0} = 1.24$	0.991
pH	$X_{2_0} = 0.95$	7.975
Temp (°C)	$X_{3_0} = -0.66$	21.6

therefore a saddle point (Myers, 1976), an example of which is illustrated in Fig. A4 (Appendix II). Since only one of the eigenvalues was positive, the canonical equation can be reduced to: $y = y_s + 0.27w_1^2$

The levels of factors required to increase CO_2 production were found using the relationship $w = M'z$ (Fig. A5, Appendix II). The levels of variables obtained for certain specified values of w_1 and the predicted response using the above relationship are shown in Table 4.8. Good agreement was again found between the predicted amounts of CO_2 production and results obtained using these levels experimentallly. It is evident from Table 4.8 that an increase in CO_2 production and, hence, reduction of the shelf life of the product would be expected at a_w 0.992, pH 7.6 and a storage temperature of 25.2°C.

After checking experimentally the accuracy of the fitted model and associated response surface to predict the amounts of CO_2 produced, contour diagrams representing various areas of the response surface were plotted.

The uncoded main effects and interactions from the CCD (Table 4.5) were used to compute a second order regression model, which was subsequently used for production of contour plots. Each contour plot was generated by varying two factors while holding a third constant. The range of each factor varied and the value of the variable held constant are shown in their respective contour plots.

TABLE 4.8 Values of coded and uncoded variables for various values of w_1 and zero values of w_2 and w_3 computed to assist in locating increased response.

	VALUES OF VARIABLES						PRODUCTION OF CO_2 AFTER 72 HRS (ml)	
	CODED			UNCODED				
w_1 Values	a_w	pH	Temp (°C)	a_w	pH	Temp (°C)	Predicted	Observed
2.42	1.37	0.15	1.62	0.992	7.63	24.3	16.7	16.4
2.44	1.37	0.14	1.64	0.992	7.6	24.9	16.75	16.4
2.48	1.37	0.13	1.66	0.992	7.6	25.2	16.8	16.6
0	1.682	0	0	0.995	7.5	22.5	16.0	15.7
0	1	1	1	0.99	8	24	16.9	16.5

The value of these plots is in their visual representation of combinations of environmental factors which would increase or decrease CO_2 production and, hence, increase or decrease shelf life. For example, in Fig. 4.1 (*aw* vs pH) a 50% increase in CO_2 production could be expected in crumpets with *aw* 0.98-0.995 and pH 7.8-8.3 stored at 20°C, compared to crumpets with *aw* 0.976-0.978 and pH 6.7-7.8 stored at the same temperature.

Storage of crumpets, with the range of *aw* and pH values shown above, at 22.5 and 25°C (Figs. 4.2,3.3) would result in a further increase in CO_2 production and a further reduction in shelf life of the product. Similar conclusions can be made on shelf life performance for various ranges of *aw* vs temperature (Figs. 4.4-4.6) and pH vs temperature (Figs. 4.7-4.9).

It is evident from Figs. 4.1-4.9 that the levels of factors chosen for the construction of each plot are all favorable to the growth and heterofermentative activity of *Leuconostoc mesenteroides*. The levels of environmental factors of interest to the manufacturer are those which could be used to control the growth of the predominant gas-producing organism. Of particular importance are the levels of *aw* and pH, which can be controlled by the manufacturer much more readily than storage temperatures. The influence of lowest levels of both *aw* and pH on growth are illustrated in Figs. 4.10-4.12. For example, CO_2 production would be completely inhibited in crumpets with *aw*

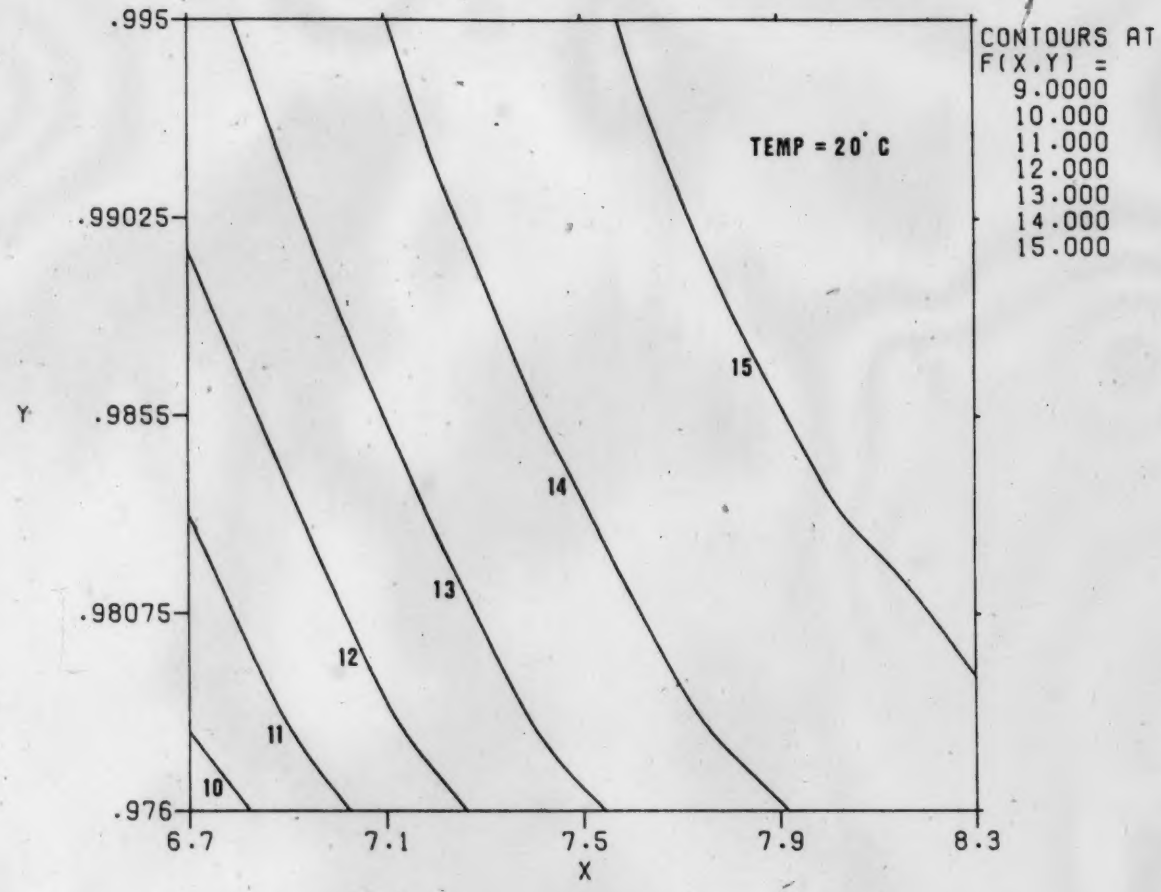


FIG. 4.1 Contour plot for CO_2 production (ml/72hr) of a_w (Y) versus pH (X) with temperature held constant at 20°C

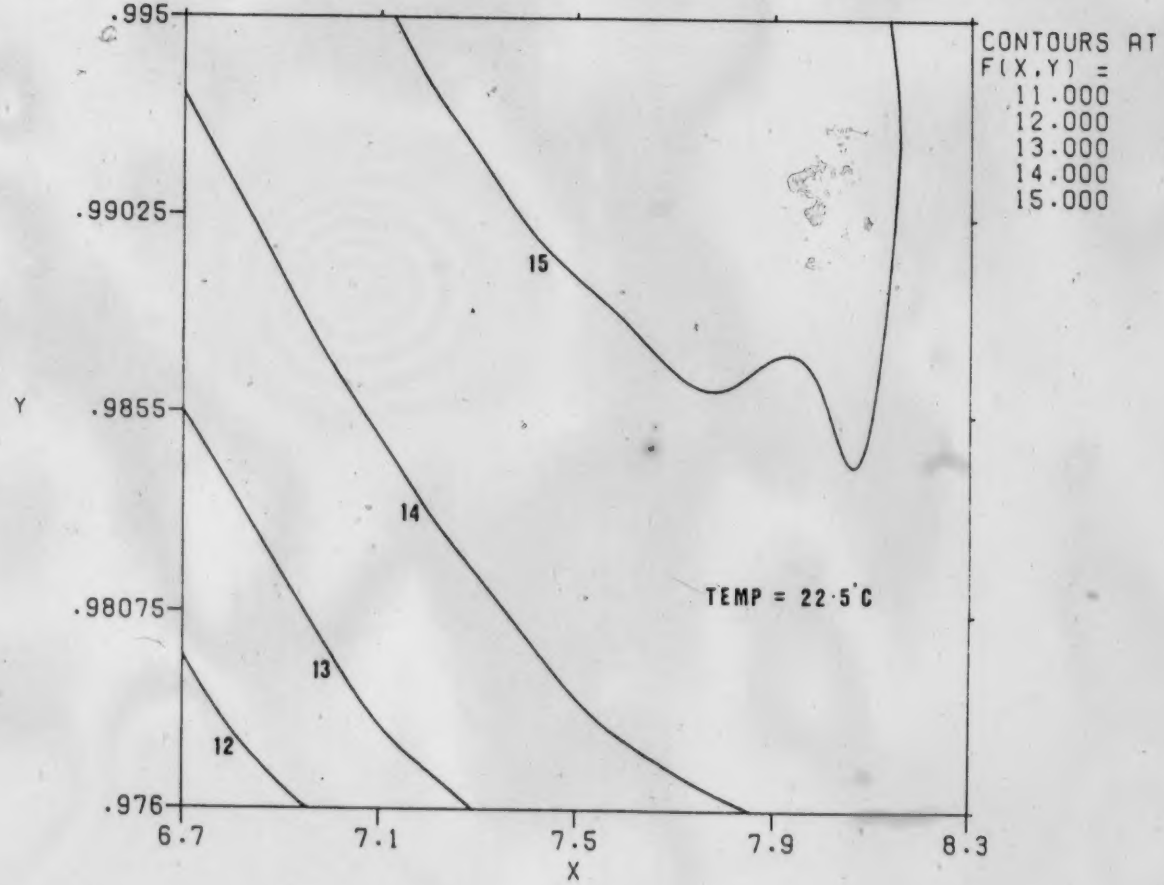


FIG. 4.2 Contour plot for CO_2 production (ml/72hr) of a_w (Y) versus pH (X) with temperature held constant at 22.5°C

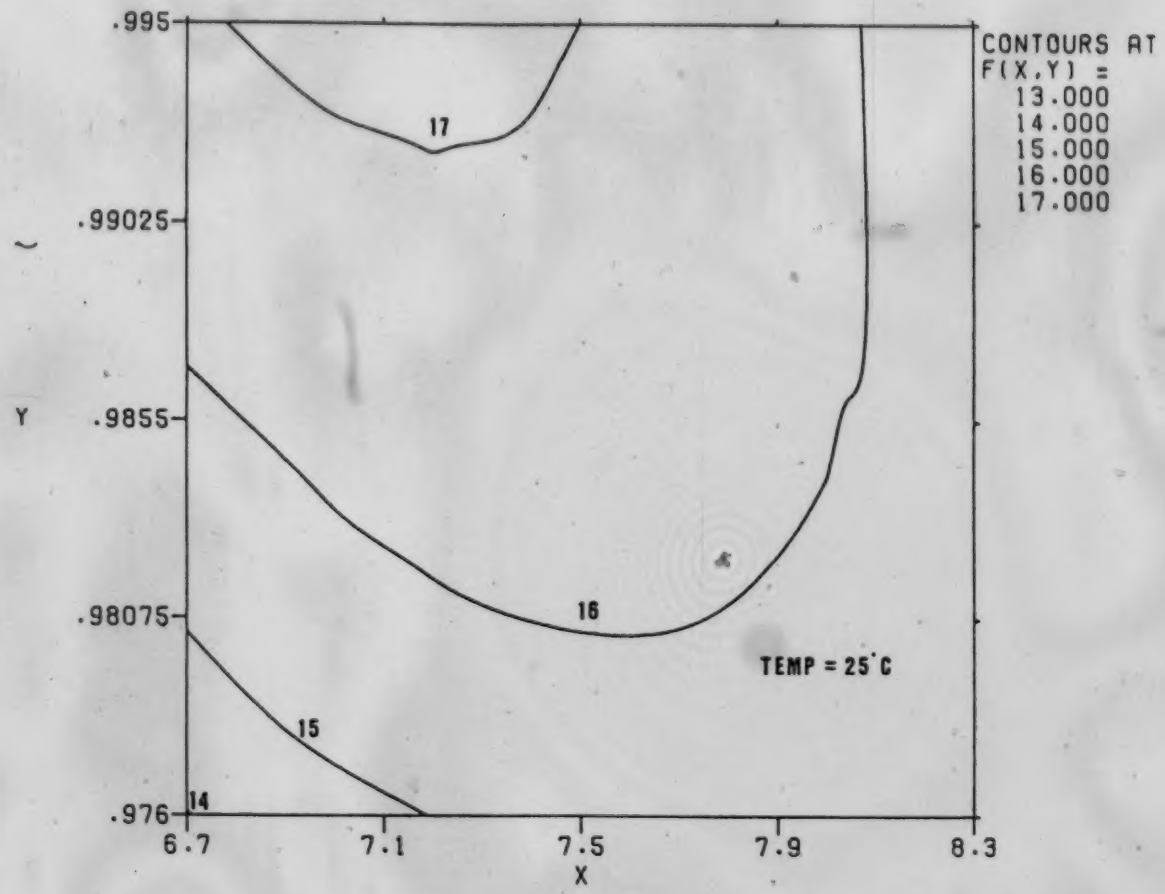


FIG. 4.3 Contour plot for CO_2 production (ml/72hr) of a_w (Y) versus pH (X) with temperature held constant at 25°C

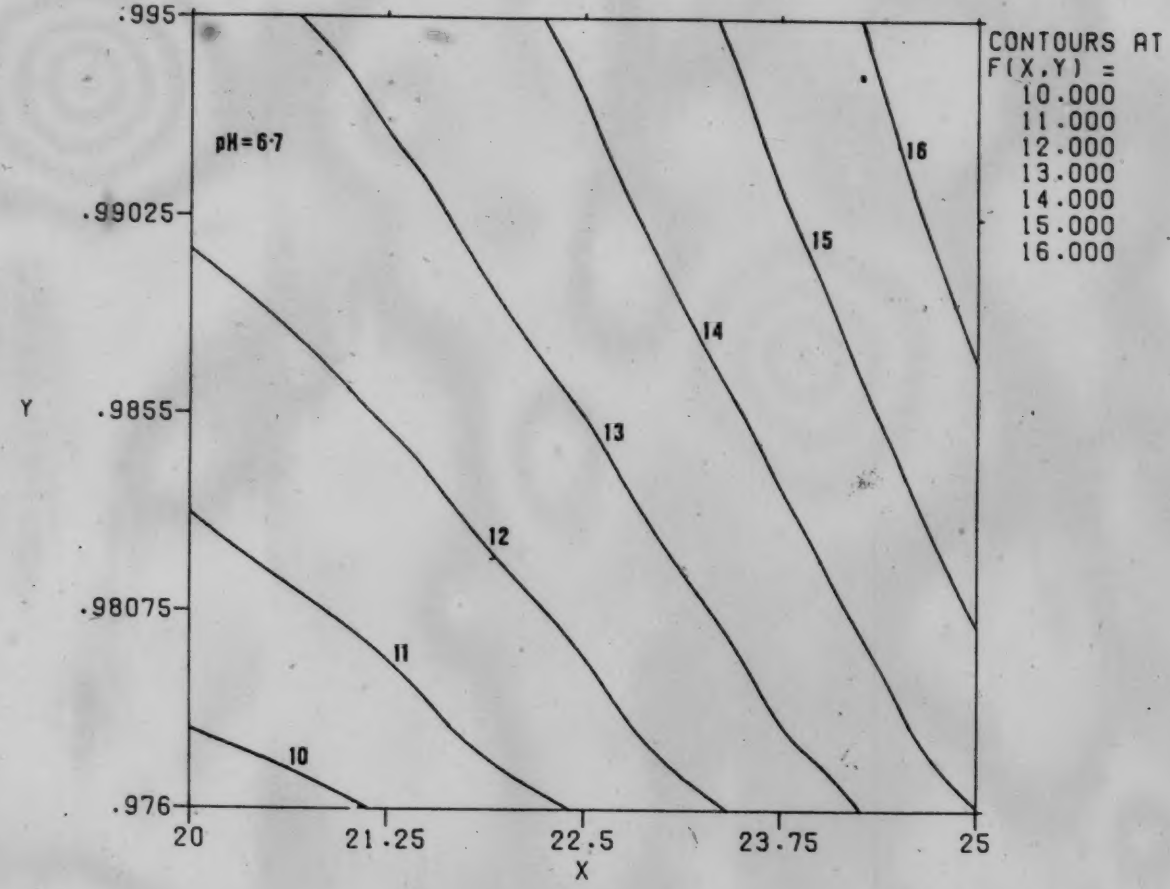


FIG. 4.4 Contour plot for CO_2 production (ml/72hr) of a_w (Y) versus temperature (X) with pH held constant at 6.7

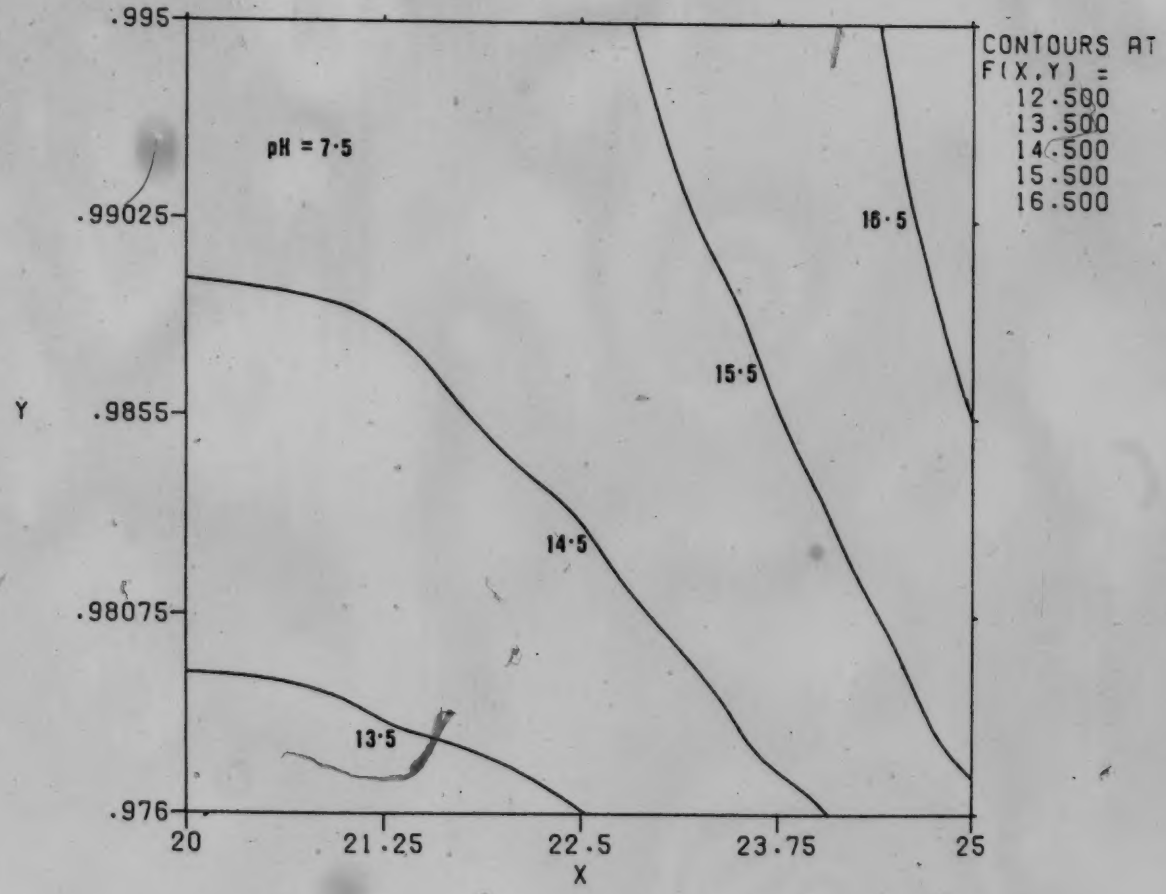


FIG. 4.5 Contour plot for CO_2 production (ml/72hr) of a_w (Y) versus temperature (X) with pH held constant at 7.5

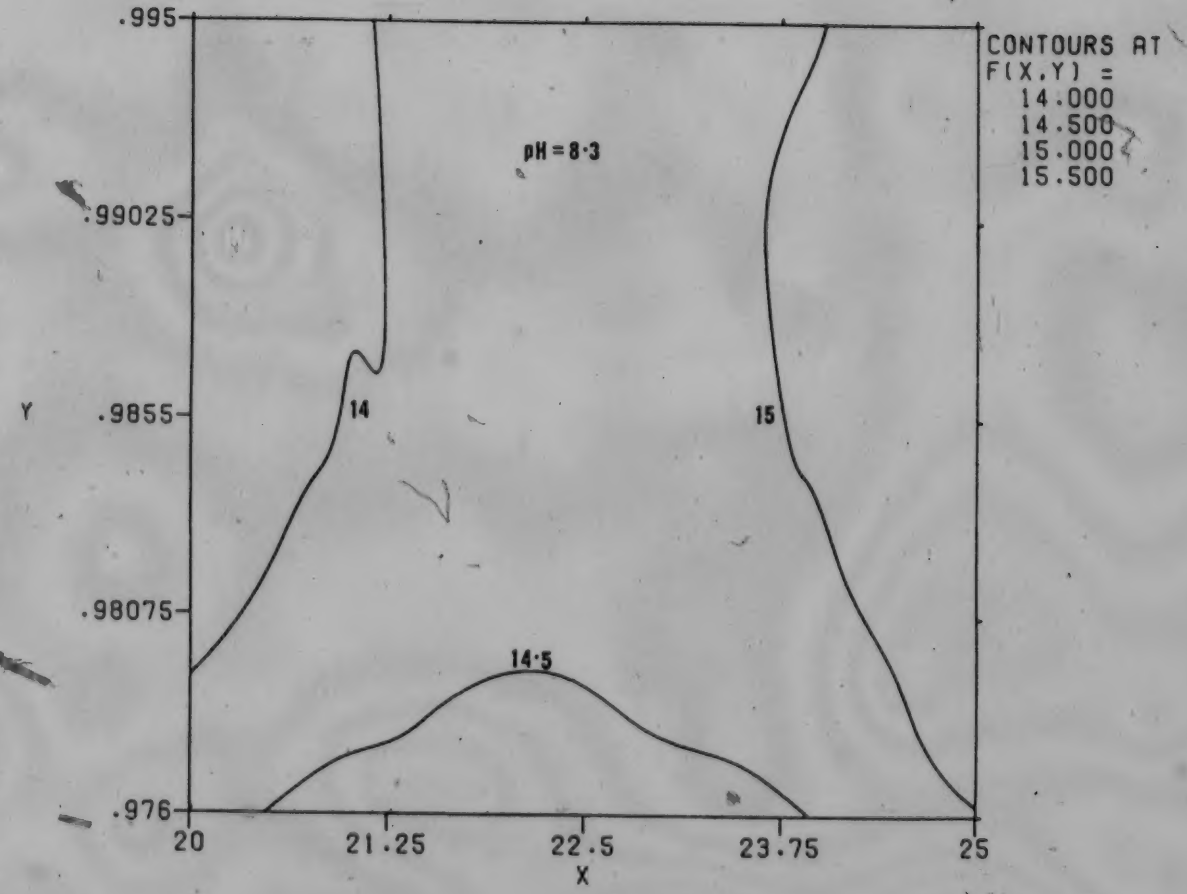


FIG. 4.6 Contour plot for CO_2 production (ml/72hr) of a_w (Y) versus temperature (X) with pH held constant at 8.3

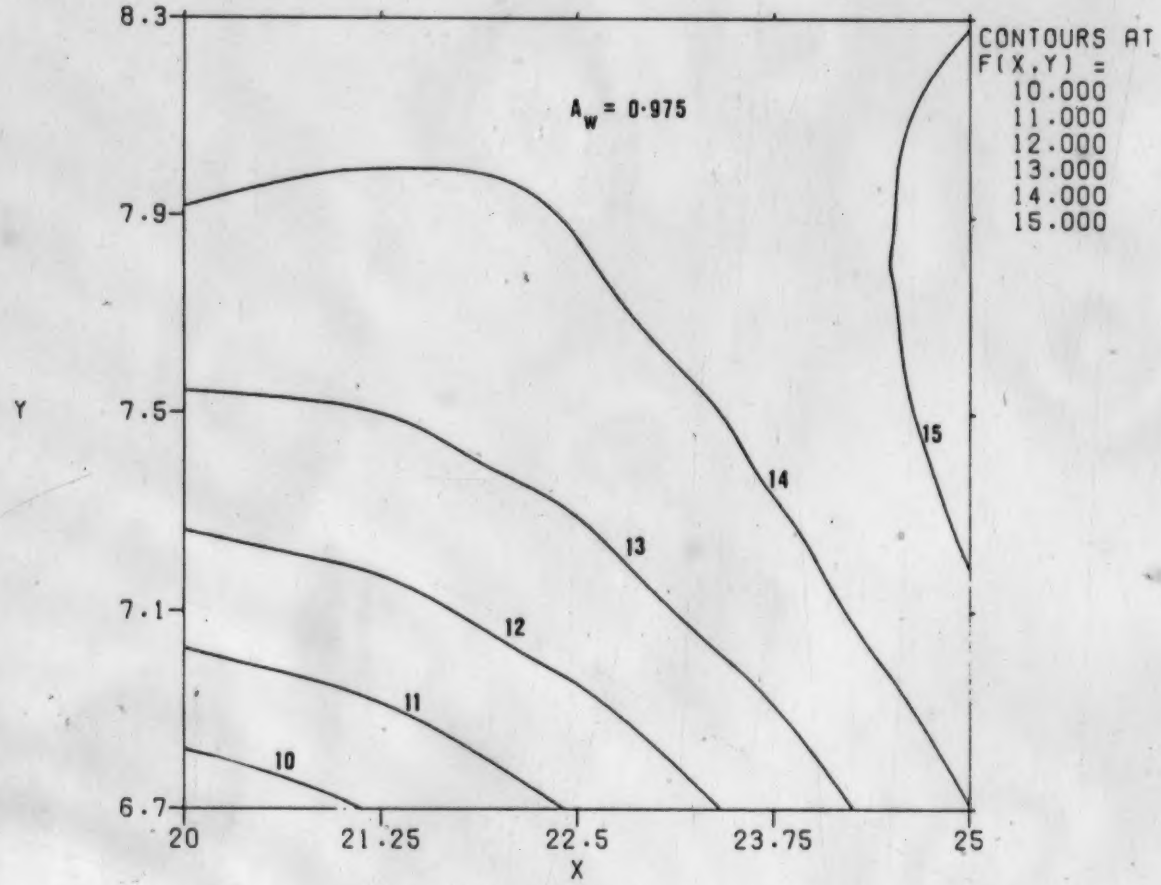


FIG. 4.7 Contour plot for CO_2 production (ml/72hr) of pH (Y) versus temperature (X) with a_w held constant at 0.975

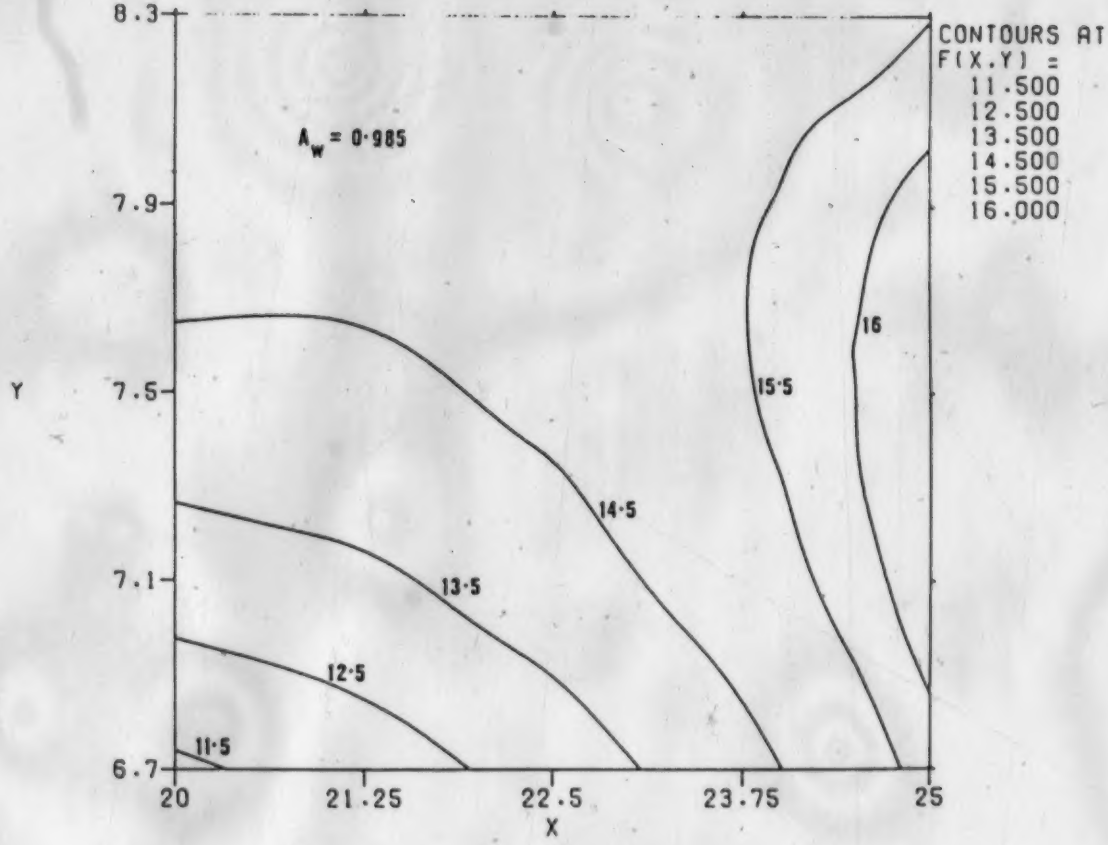


FIG. 4.8 Contour plot for CO_2 production (ml/72hr) of pH (Y) versus temperature (X) with a_w held constant at 0.985

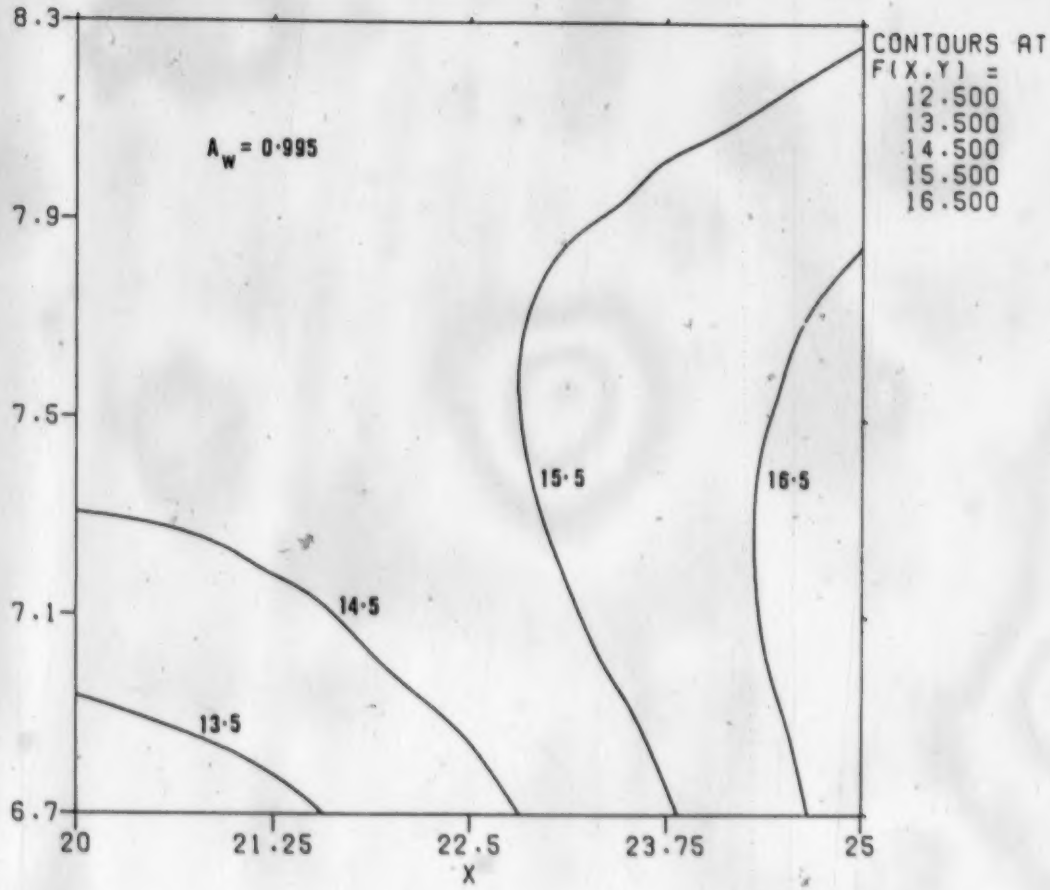


FIG. 4.9 Contour plot for CO_2 production (ml/72hr) of pH (Y) versus temperature (X) with a_w held constant at 0.995

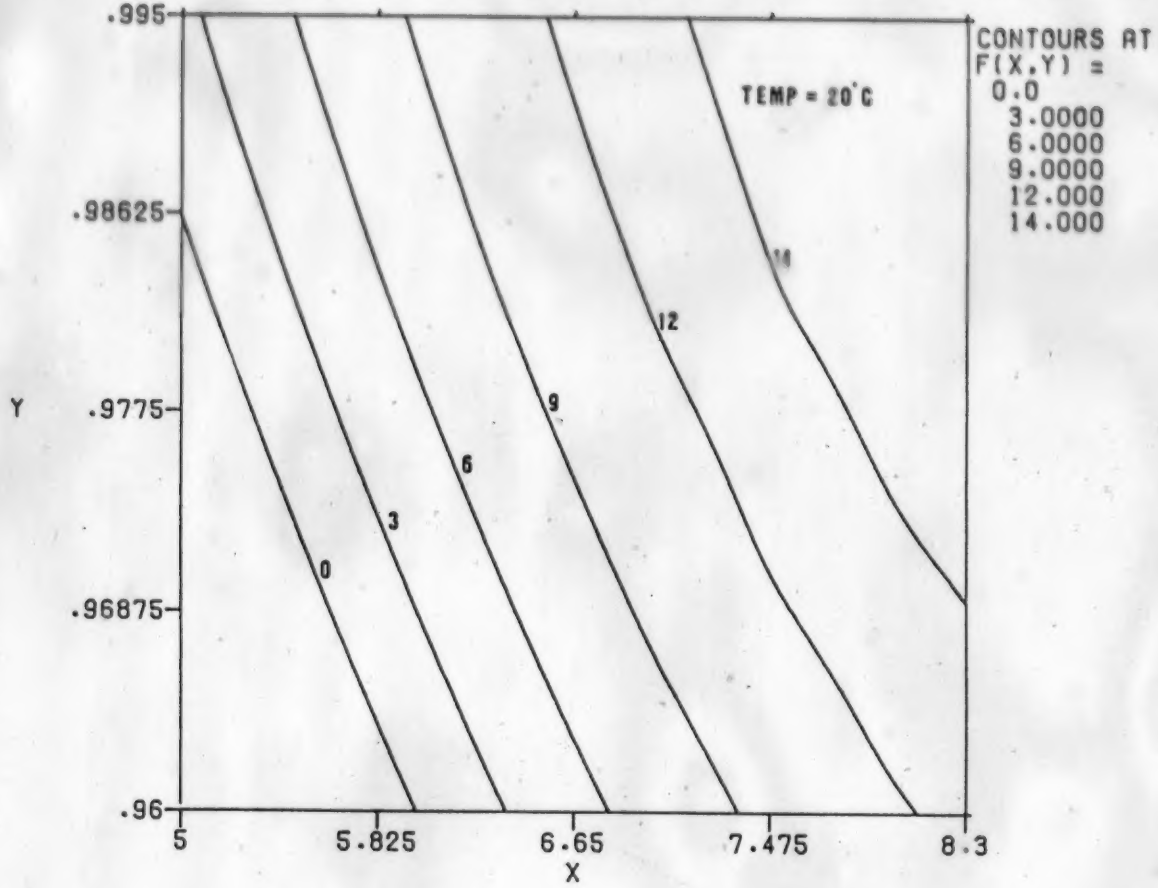


FIG. 4.10 Contour plot for CO_2 production (ml/72hr) of lower levels of a_w (Y) versus lower pH values (X) with temperature held constant at 20°C

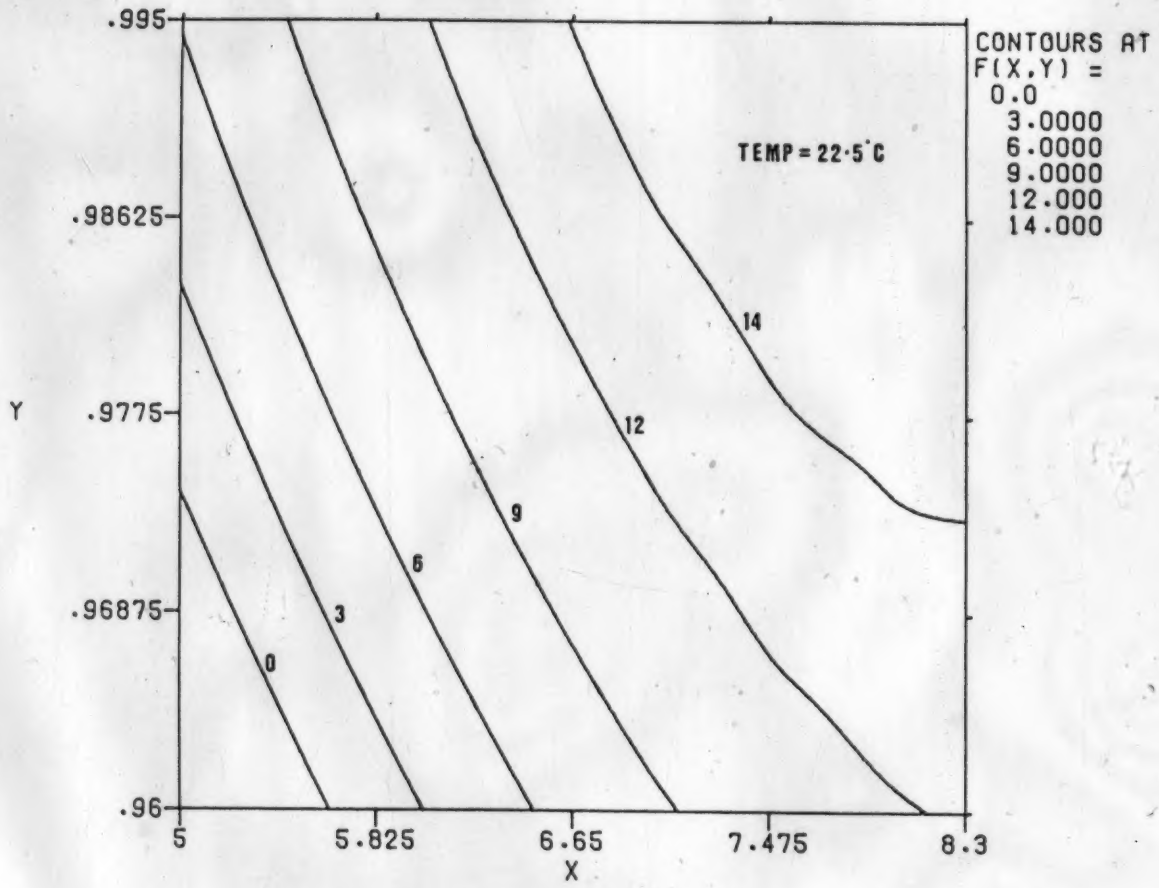


FIG. 4.11 Contour plot for CO_2 production (ml/72hr) of lower levels of a_w (Y) versus lower pH values (X) with temperature held constant at 22.5°C

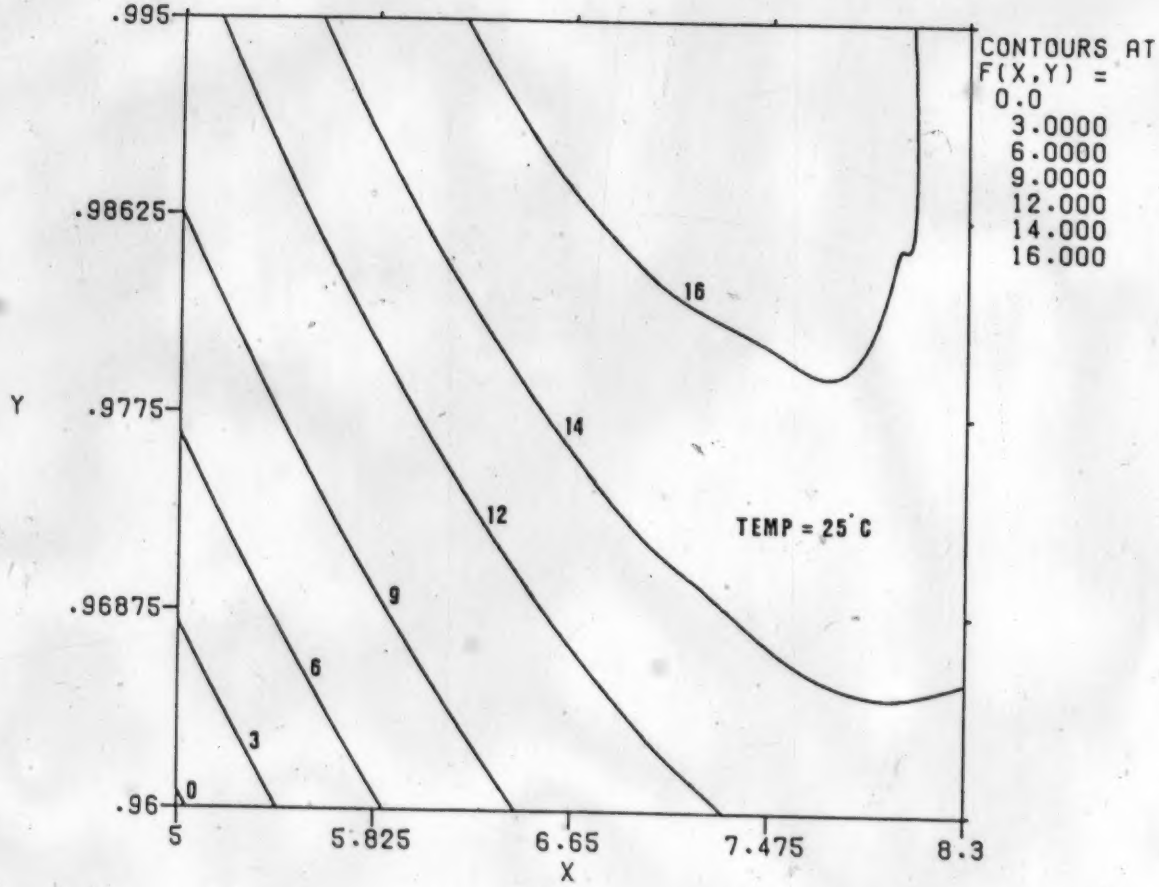


FIG. 4.12 Contour plot for CO_2 production (ml/72hr) of lower levels of a_w (Y) versus lower pH values (X) with temperature held constant at 25°C

0.96-0.98 and pH 5 stored at 20°C (Fig. 4.10). Even at higher levels of *aw* (0.96-0.995) and pH (5.4 or less), CO_2 production could still be substantially reduced at 20°C. At slightly higher storage temperatures (22.5°C) the manufacturer would need to reformulate the product to *aw* 0.96 and pH 5.7 or less to ensure minimum gas production (Fig. 4.11). However, if higher *aw* levels were used (0.98), the pH must be reduced to 5.3 to inhibit CO_2 production.

At normal retail storage temperatures (25°C), CO_2 production could still be controlled by reformulation of the product to *aw* 0.96-0.97 and pH 5 (Fig. 4.12).

The predicted levels of CO_2 at lower *aw* and pH agree favorably with actual results (Table 4.9) obtained in earlier studies with *Leuconostoc mesenteroides*. Therefore, by referring to the contour plots, the manufacturer can choose, at his discretion, combinations of high pH-low *aw* or low pH-high *aw* to reformulate the product and so extend shelf life performance.

4.3.2 (b) *Aspergillus niger*

Similar results to those obtained for *Leuconostoc mesenteroides* were found in the initial 2^{4-3} screening design (Table 4.10) for *Aspergillus niger*, namely the low *aw* level (0.85) masked the effect of all other factors and, consequently, *aw* was the only factor of significance. These results were unexpected, considering the xerophilic nature of *Aspergillus niger*, which has been reported to grow at *aw*

TABLE 4.9 Influence of selected levels of a_w, pH and temperature on CO_2 production by *Leuconostoc mesenteroides*

Runs 1-3 - First Order Polynomial Design (Table 3.3)

Runs 4-6 - Path of Steepest Ascent Approach (Table 3.4)

a_w	pH	TEMP (°C)	CO_2 PRODUCTION (ml)
0.97	5.75	20	3.5
0.97	5.25	20	0
0.97	5.25	30	0
0.97	5.25	25	0
0.975	5.25	20	1.85
0.975	5.75	25	3.7

TABLE 4.10 A 2^{6-3} factorial screening design to determine factors influencing the growth of *Aspergillus*

a_w	pH	INOCULUM LEVEL SPORES/PLATE	CONC. OF INHIBITOR (%)	CO_2:AIR RATIO(%)	TEMP(°C)	DAYS TO VISIBLE GROWTH
0.85	5	$2x10^2$	0.13	50	20	> 28
0.95	5	$2x10^2$	0.065	0	30	2.5
0.85	7	$2x10^2$	0.065	50	30	> 28
0.95	7	$2x10^2$	0.13	0	20	4
0.85	5	$2x10^3$	0.13	0	30	> 28
0.95	5	$2x10^3$	0.065	50	20	12.5
0.85	7	$2x10^3$	0.065	0	20	> 28
0.95	7	$2x10^3$	0.13	50	30	7.5

VARIABLES	LEVELS -1	0	+1
a_w (X_1)	0.85	0.9	0.95
pH (X_2)	5	6	7
Inoculum(spores/plate) (X_3)	$2x10^2$	$11x10^2$	$2x10^3$
Conc. of inhib.(%) (X_4)	0.065	0.095	0.13
CO_2:air ratio (%) (X_5)	0:100	25:75	50:50
Temp. (°C) (X_6)	20	25	30

0.8-0.82 when all other environmental conditions are near optimum (Troller and Christian, 1980; Banwart, 1979). This stresses the contention made earlier that one factor at a time experimentation may exaggerate the levels of specific environmental factors necessary to control growth.

To determine the influence of other environmental factors on growth and compute the linear regression model, a first order design (Table 4.11), with a more restricted range of a_w values (0.94-0.98), was performed. The regression equation obtained from analysis of the data was:

$$y = \beta_0 - 101.56X_1 - 0.59X_2 - 0.0002X_3 + 18.27X_4 + 0.0625X_5 - 0.2X_6$$

Examination of the fitted coefficients using Student's two-tailed t-test (24 d.f.) showed that all factors, with the exception of inoculum levels, were significant. The lack of significance of inoculum level was surprising, as it was assumed that the greater the level of spores contaminating the surface of the product, the more likely mold problems would occur. However, the results obtained do agree with Seiler (1977), who reported in studies with high ratio Madeira cake that inoculum level had little or no effect on the mold-free shelf life of the product.

The significance of a_w, CO_2 and inhibitor is also in agreement with the work of Seiler (1978), who found that these factors could be used to inhibit or suppress mold growth on cake and bread.

TABLE 4.11 A $2^2 \times 2^{6-3}$ design to determine factors influencing the growth of *Aspergilles niger* and compute first order polynomial model

BLOCK	a_w	pH	INOCULUM LEVEL SPORES/PLATE	CONC. OF INHIBITOR (%)	CO_2:AIR RATIO(%)	TEMP (°C)	DAYS TO VISIBLE GROWTH
-1	0.96	5	$2x10^2$	0.13	50	20	8.25
-1	0.98	5	$2x10^2$	0.065	0	30	1.5
-1	0.96	7	$2x10^2$	0.065	50	30	5
-1	0.98	7	$2x10^2$	0.13	0	20	2.75
-1	0.96	5	$2x10^3$	0.13	0	30	3.25
-1	0.98	5	$2x10^3$	0.065	50	20	5.5
-1	0.96	7	$2x10^3$	0.065	0	20	4
-1	0.98	7	$2x10^2$	0.13	50	30	4.5
1	0.96	5	$2x10^2$	0.13	50	20	8.25
1	0.98	5	$2x10^2$	0.065	0	30	1.75
1	0.96	7	$2x10^2$	0.065	50	30	5.5
1	0.98	7	$2x10^2$	0.13	0	20	3
1	0.96	5	$2x10^3$	0.13	0	30	3.5
1	0.98	5	$2x10^3$	0.065	50	20	6
1	0.96	7	$2x10^3$	0.065	0	20	4.5
1	0.98	7	$2x10^3$	0.13	50	30	5
-1	0.94	5	$2x10^2$	0.13	50	20	14
-1	0.96	5	$2x10^2$	0.065	0	30	2.75
-1	0.94	7	$2x10^2$	0.065	50	30	5.75
-1	0.96	7	$2x10^2$	0.13	0	20	4.25
-1	0.94	5	$2x10^3$	0.13	0	30	5.75
-1	0.96	5	$2x10^3$	0.065	50	20	6.75
-1	0.94	7	$2x10^3$	0.065	0	20	5.75
-1	0.96	7	$2x10^2$	0.13	50	30	5.25
1	0.94	5	$2x10^2$	0.13	50	20	12
1	0.96	5	$2x10^2$	0.065	0	30	3
1	0.94	7	$2x10^2$	0.065	50	30	6
1	0.96	7	$2x10^3$	0.13	0	20	4.5
1	0.94	5	$2x10^3$	0.13	0	30	5.5
1	0.96	5	$2x10^3$	0.065	50	20	7.5
1	0.94	7	$2x10^3$	0.065	0	20	5
1	0.96	7	$2x10^3$	0.13	50	30	5.5

To determine the levels of factors required to increase response, a path of steepest ascent approach was used. It is again evident from the signs of the regression coefficients that, as *a*w, pH and storage temperature decrease and level of inhibitor and CO_2 concentration increase, the number of days to detect visible mold growth will also increase. The two path of steepest ascent designs, with *a*w held constant at 0.98 and differing only in the addition of inhibitor (Tables 4.12-4.13) indeed confirm the expected trend. The predicted values shown in Tables 4.12-4.13 were obtained as previously outlined for the path of steepest ascent approach for *Leuconostoc mesenteroides*. Good agreement was again found between these values and actual results obtained experimentally, with the exception of RUN 18 in Table 4.13. Here, the actual days to grow were much greater than the predicted values.

A possible reason for the discrepancy for RUN 18 could be an interaction between temperature and CO_2, which would reduce pH as a result of the dissolution of CO_2 into the medium at lower storage temperatures. Such a reduction in pH would enhance the antimycotic activity of potassium sorbate, which has been shown to be more effective at lower pH values (Sauer, 1977).

To test this hypothesis a CCD for k=4, with pH held constant at 6, was carried out. The construction of a CCD for k=4 is shown in Table A12 (Appendix II) with the uncoded values actually used in experimental runs shown in Table

TABLE 4.12 Days to visible mold growth with variable levels determined using the path of steepest ascent approach

	LEVELS			DAYS TO VISIBLE GROWTH	
CO_2:Air ratio(%)	a_w	Temp (°C)	pH	Predicted	Observed
30	0.98	25	5.92	2.04	2
35	0.98	25	5.85	2.40	2
40	0.98	25	5.78	2.75	2
45	0.98	25	5.71	3.11	2.5
50	0.98	25	5.64	3.46	3
55	0.98	25	5.56	3.82	3
60	0.98	25	5.49	4.17	3.5
65	0.98	25	5.42	4.53	3.75
70	0.98	25	5.35	4.88	4
30	0.98	20	5.92	3.06	3
35	0.98	20	5.85	3.41	3
40	0.98	20	5.78	3.77	3
45	0.98	20	5.71	4.12	3.5
50	0.98	20	5.64	4.48	3.75
55	0.98	20	5.56	4.83	4
60	0.98	20	5.49	5.19	4.25
65	0.98	20	5.42	5.54	4.75
70	0.98	20	5.35	5.90	6.5

TABLE 4.13 Days to visible mold growth with variable levels determined using the path of steepest ascent approach

LEVELS					DAYS TO VISIBLE GROWTH	
CO_2:Air ratio(%)	a_w	Temp (°C)	pH	Conc. of inhibitor (%)	Predicted	Observed
30	0.98	25	5.92	0.09	3.86	3
35	0.98	25	5.85	0.10	4.25	3.25
40	0.98	25	5.78	0.10	4.65	3.75
45	0.98	25	5.71	0.10	5.04	4.25
50	0.98	25	5.64	0.10	5.43	4.5
55	0.98	25	5.56	0.10	5.83	5
60	0.98	25	5.49	0.11	6.22	5.5
65	0.98	25	5.42	0.11	6.61	5.75
70	0.98	25	5.35	0.11	7.01	6.25
30	0.98	20	5.92	0.09	4.88	4.25
35	0.98	20	5.85	0.10	5.27	4.25
40	0.98	20	5.78	0.10	5.66	5.25
45	0.98	20	5.71	0.10	6.06	5.25
50	0.98	20	5.64	0.10	6.45	5.75
55	0.98	20	5.56	0.10	6.84	6
60	0.98	20	5.49	0.11	7.24	7.75
65	0.98	20	5.42	0.11	7.63	9.5
70	0.98	20	5.35	0.11	8.02	15

4.14. The levels of α were from Myers (1976). The second order regression equation obtained from analysis of the coded data (Table 4.15) was:

$$y = \beta_0 - 4.4X_1 + 0.86X_2 - 5.0X_3 + 2.9X_4 + 2.1X_1^2 + 0.45X_2^2 + 0.83X_3^2 + 0.8X_4^2 - 0.5X_1X_2 + 2.9X_1X_3 - 2X_1X_4 - 0.31X_2X_3 + 0.3X_2X_4 - 2.3X_3X_4$$

Examination of the fitted model using Student's t-test (15 d.f.) indicated regression coefficients for *aw*, temperature and CO_2 (X_1, X_3 and X_4) and *aw*.T (X_1X_3), *aw*.CO_2 (X_1X_4) and T.CO_2 (X_3X_4) were significant. Subsequent analysis of the stationary point (X_0) generated from the above data revealed that the stationary point for CO_2:Air ratio was well outside the experimental range. Subsequently, a reduced second order regression was computed using only the significant terms in Table 4.15.

The reduced second order equation was:

$$y = \beta_0 - 4.4X_1 - 5X_3 + 2.9X_4 + 2.9X_1X_3 - 2X_1X_4 - 2.3X_3X_4$$

All terms in the model were significant (18 d.f.). Values of *aw*, temperature and CO_2:Air ratio at the stationary point are shown in Table 4.16 and are well within the range of experimental levels. A predicted shelf life of 5.5 days (y_0) was found for levels at the stationary point.

Analysis of the canonical form of the fitted equation, i.e.

$$y = y_0 + 2.4w_1^2 - 0.94w_2^2 - 1.45w_3^2$$

indicated a similar type of response surface to that obtained for *Leuconostoc mesenteroides* (a saddle point). As

TABLE 4.14 A Central Composite Design for k=4 for development of second order polynomial model

a_w	CONC. OF INHIBITOR (%)	TEMP (°C)	CO_2:AIR RATIO(%)	DAYS TO VISIBLE GROWTH
0.945	0.075	21	53	14
0.975	0.075	21	53	7
0.945	0.12	21	53	16.5
0.975	0.12	21	53	8
0.945	0.075	24	53	5.5
0.975	0.075	24	53	3
0.945	0.12	24	53	6.5
0.975	0.12	24	53	3.5
0.945	0.075	21	67	31
0.975	0.075	21	67	10.5
0.945	0.12	21	67	36
0.975	0.12	21	67	12
0.945	0.075	24	67	7
0.975	0.075	24	67	4
0.945	0.12	24	67	10
0.975	0.12	24	67	4.5
0.98	0.0975	22.5	60	6
0.94	0.0975	22.5	60	16
0.96	0.13	22.5	60	8.5
0.96	0.065	22.5	60	7
0.96	0.0975	25	60	5
0.96	0.0975	20	60	12
0.96	0.0975	22.5	70	11
0.96	0.0975	22.5	50	6
0.96	0.0975	22.5	60	7.5

TABLE 4.15 Main effects and interactions used to compute second order model for *Aspergillus niger*

a_w	Inh	Temp	Co_2:Air ratio	a_w^2	Inh^2	$Temp^2$	CO_2^2	a_w.Inh	Aw.T	Aw.CO_2	Inh.Temp	Inh.CO_2	Temp.CO_2	
-1	-1	-1	-1	1	1	1	1	1	1	1	1	1	1	14
1	-1	-1	-1	1	1	1	1	-1	-1	-1	1	1	1	7
-1	1	-1	-1	1	1	1	1	-1	1	1	-1	-1	1	16.5
1	1	-1	-1	1	1	1	1	1	-1	-1	-1	-1	1	8
-1	-1	1	-1	1	1	1	1	1	-1	1	-1	1	-1	5.5
1	-1	1	-1	1	1	1	1	-1	1	-1	-1	1	-1	3
-1	1	1	-1	1	1	1	1	-1	-1	1	1	-1	-1	6.5
1	1	1	-1	1	1	1	1	1	1	-1	1	-1	-1	3.5
-1	-1	-1	1	1	1	1	1	1	1	-1	1	-1	-1	31
1	-1	-1	1	1	1	1	1	-1	-1	1	1	-1	-1	10.5
-1	1	-1	1	1	1	1	1	-1	1	-1	-1	1	-1	36
1	1	-1	1	1	1	1	1	1	-1	1	-1	1	-1	12
-1	-1	1	1	1	1	1	1	1	-1	-1	-1	-1	1	7
1	-1	1	1	1	1	1	1	-1	1	1	-1	-1	1	4
-1	1	1	1	1	1	1	1	-1	-1	-1	1	1	1	10
1	1	1	1	1	1	1	1	1	1	1	1	1	1	4.5
1.42	0	0	0	2.01	0	0	0	0	0	0	0	0	0	6
-1.42	0	0	0	2.01	0	0	0	0	0	0	0	0	0	16
0	1.42	0	0	0	2.01	0	0	0	0	0	0	0	0	8.5
0	-1.42	0	0	0	2.01	0	0	0	0	0	0	0	0	7
0	0	1.42	0	0	0	2.01	0	0	0	0	0	0	0	5
0	0	-1.42	0	0	0	2.01	0	0	0	0	0	0	0	12
0	0	0	1.42	0	0	0	2.01	0	0	0	0	0	0	11
0	0	0	-1.42	0	0	0	2.01	0	0	0	0	0	0	6
0	0	0	0	0	0	0	0	0	0	0	0	0	0	7.5

	LEVELS				
VARIABLES	-1.42($-\alpha$)	-1	0	+1	1.42 (α)
a_w (X_1)	0.94	0.945	0.96	0.975	0.98
Conc. of inh.(%)(X_2)	0.065	0.075	0.0975	0.12	0.13
Temp(°C)(X_3)	20	21	22.5	24	25
CO_2:Air ratio(%)(X_4)	50:50	53:47	60:40	67:63	70:30

TABLE 4.16 Values of coded and uncoded variables at the stationary point

VARIABLES	LEVELS	
	Coded	Uncoded
a_w	$X_1 = 0.72$	0.97
Temp (°C)	$X_3 = 0.63$	23.4
CO_2	$X_4 = -1.29$	50.9

only one of the eigenvalues was positive, the canonical equation can be reduced to:

$$y = y_0 + 2.4w_1$$

Values of coded and uncoded variables for various values of w_1, computed form the relationship w=M'Z (Fig. A5, Appendix II) to increase response, are shown in Table 4.17.

Using the above equation, a predicted shelf life of 32 days was obtained without added inhibitor for *aw* 0.94, temperature 20.4°C and CO_2:Air ratio 63.7:36.3. The actual value found experimentally, using similar levels of each factor, was 31-36 days.

Contour plots were drawn as described previously for *Leuconostoc mesenteroides*. Three sets of plots are shown:

(1) *aw* versus temperature; CO_2 concentration constant (Figs. 4.13-4.15).

(2) *aw* versus CO_2 concentration; temperature constant (Figs. 4.16-4.18).

(3) CO_2 concentration versus temperature; *aw* constant (Figs. 4.19-4.21).

By referring to these plots, the manufacturer can select levels of factors necessary to inhibit mold growth and so optimize shelf life. For example, if a mold-free shelf life of 25 days is required without added inhibitor in the product and complete inhibition of CO_2-producing lactic acid bacteria, the combination of environmental factors, summarised in Table 4.18, could be employed.

TABLE 4.17 Values of coded variables for various of w_1 and zero values of w_2 and w_3 computed to assist in locating increased response

	w_1 VALUES			
VARIABLES	-3.1	-3.2	-3.3	-3.4
a_w (X_1)	-1.1	-1.159	-1.218	-1.277 (0.941)*
Temp (°C) (X_3)	-1.249	-1.31	-1.371	-1.432 (20.37)*
CO_2:Air ratio (%) (X_4)	0.372	0.426	0.479	0.533 (63.7)*

*Uncoded values

TABLE 4.18 Levels of a_w, CO_2 and storage temperature required to give a mold free shelf life of 25 days

a_w	CO_2 CONC. (%)	STORAGE TEMP (°C)	CONTOUR PLOT
0.94	60	21	FIG. 3.19
0.95	60	20	FIG. 3.14
0.958	66	20	FIG. 3.16
0.96	68	20	FIG. 3.20
0.965	70	20	FIG. 3.15

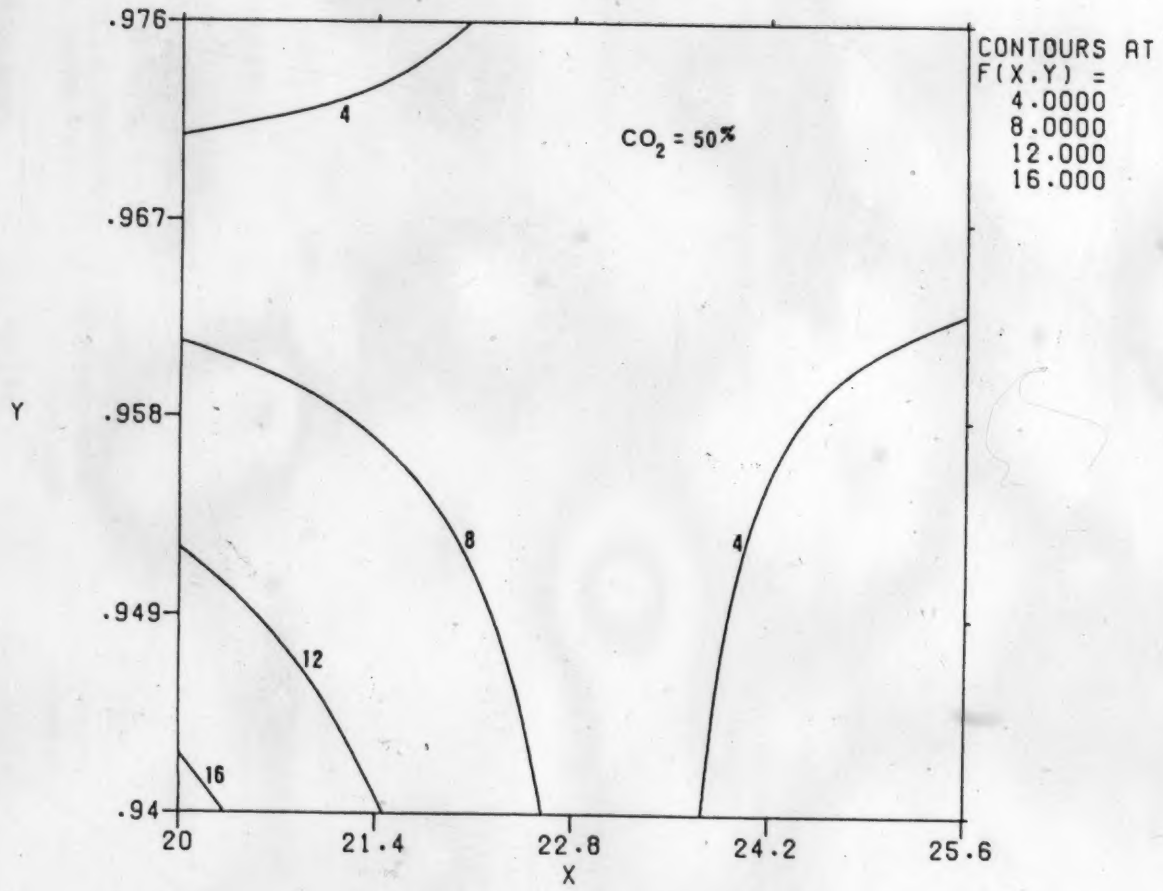


FIG. 4.13 Contour plot of days to visible mold growth for a_w(Y) versus temperature (°C)(X) with CO_2 held constant at 50%.

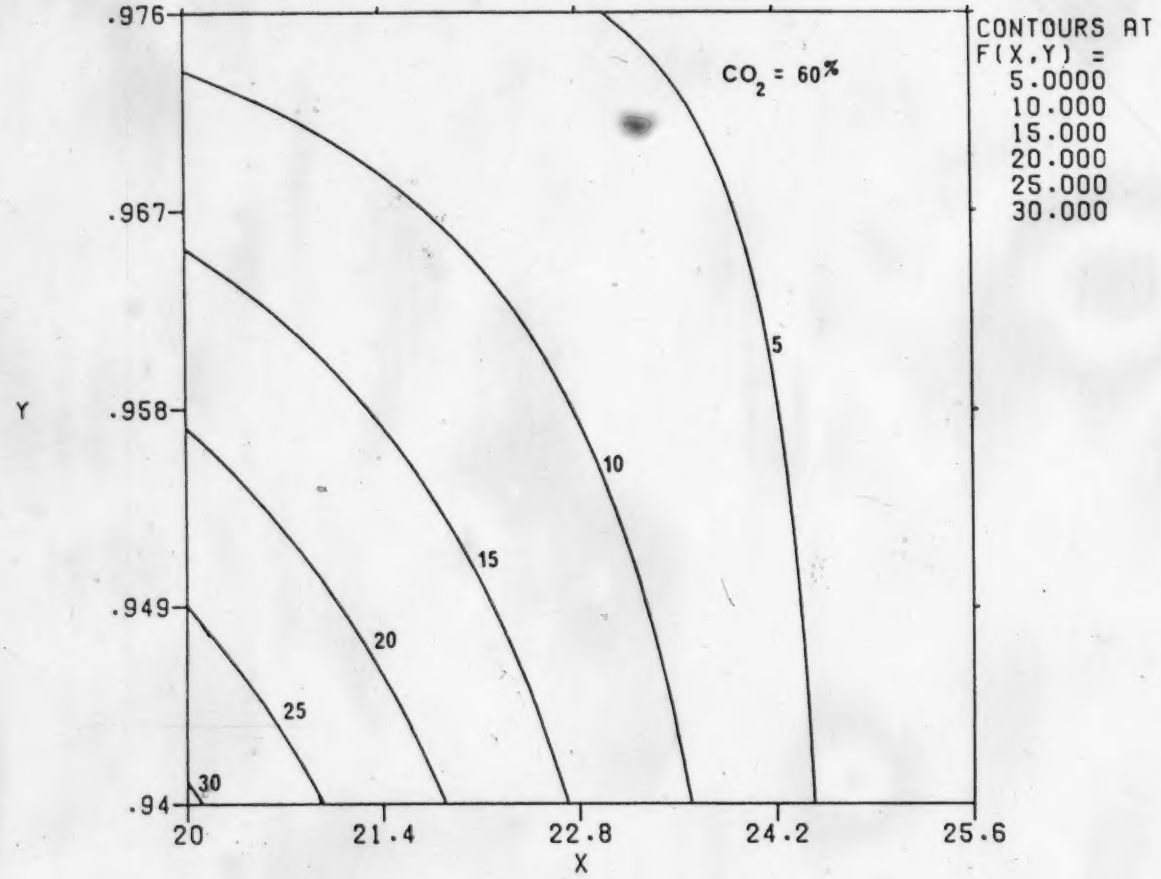


FIG. 4.14 Contour plot of days to visible mold growth for a_w(Y) versus temperature (°C) (X) with CO_2 held constant at 60%.

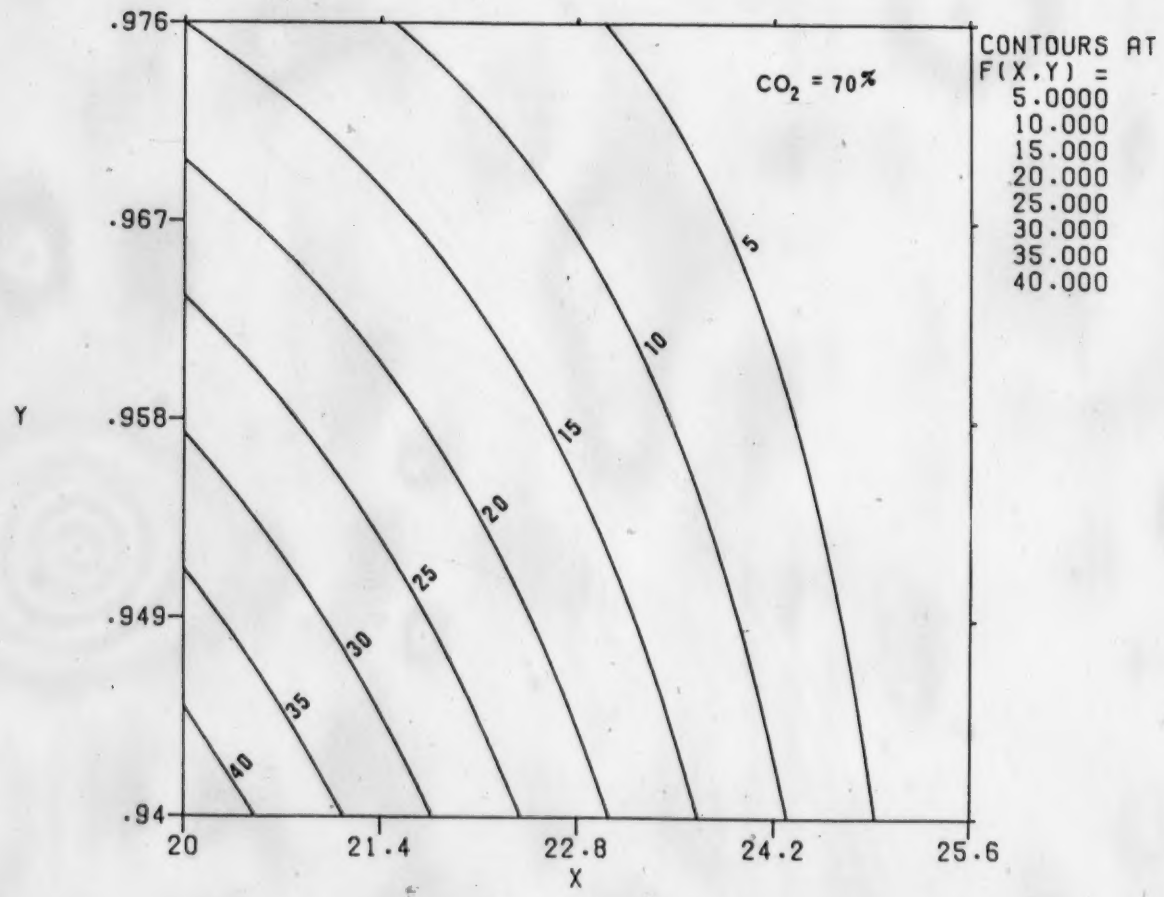


FIG. 4.15 Contour plot of days to visible mold growth for a_w(Y) versus temperature (°C)(X) with CO_2 held constant at 70%.

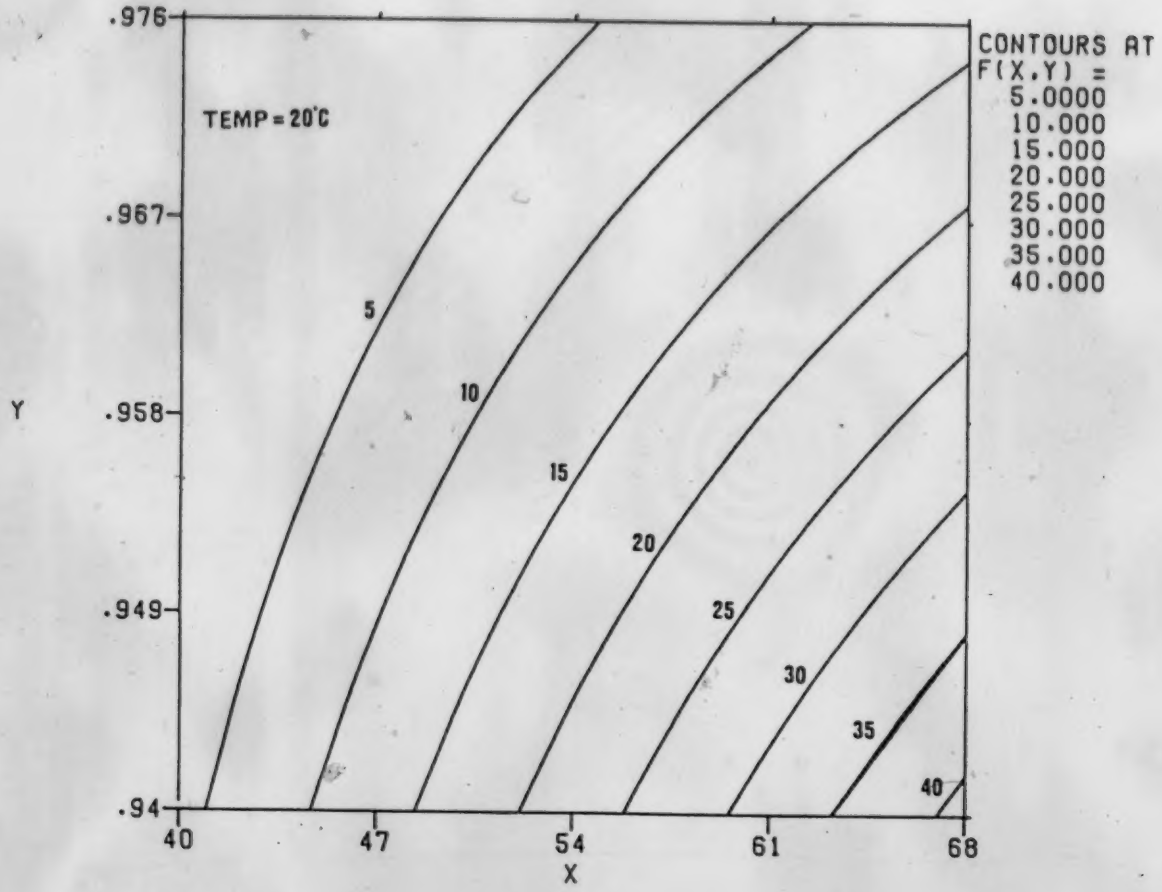


FIG. 4.16 Contour plot of days to visible mold growth for a_w (Y) versus CO_2 concentration (%)(X) with temperature held constant at 20°C.

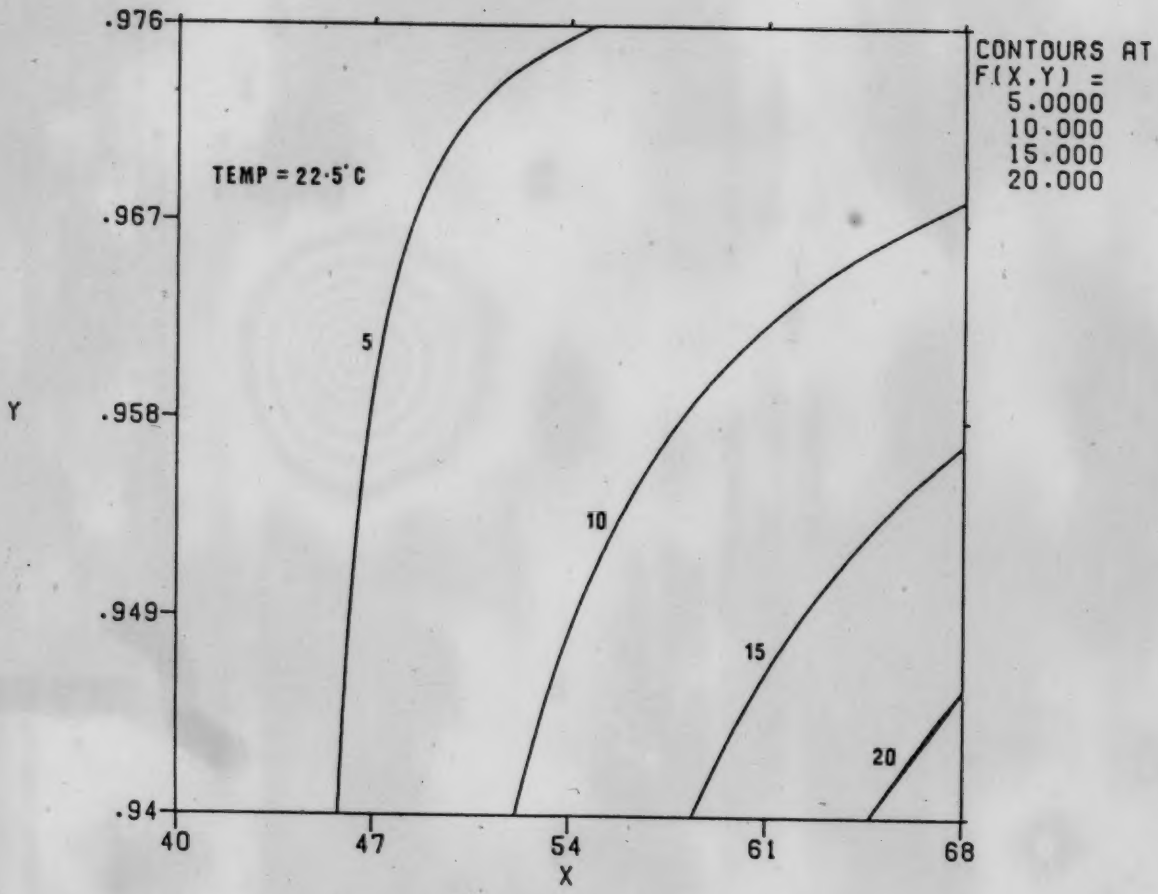


FIG. 4.17 Contour plot of days to visible mold growth for a_w(Y) versus CO_2 concentration (%)(X) with temperature held constant at 22.5°C.

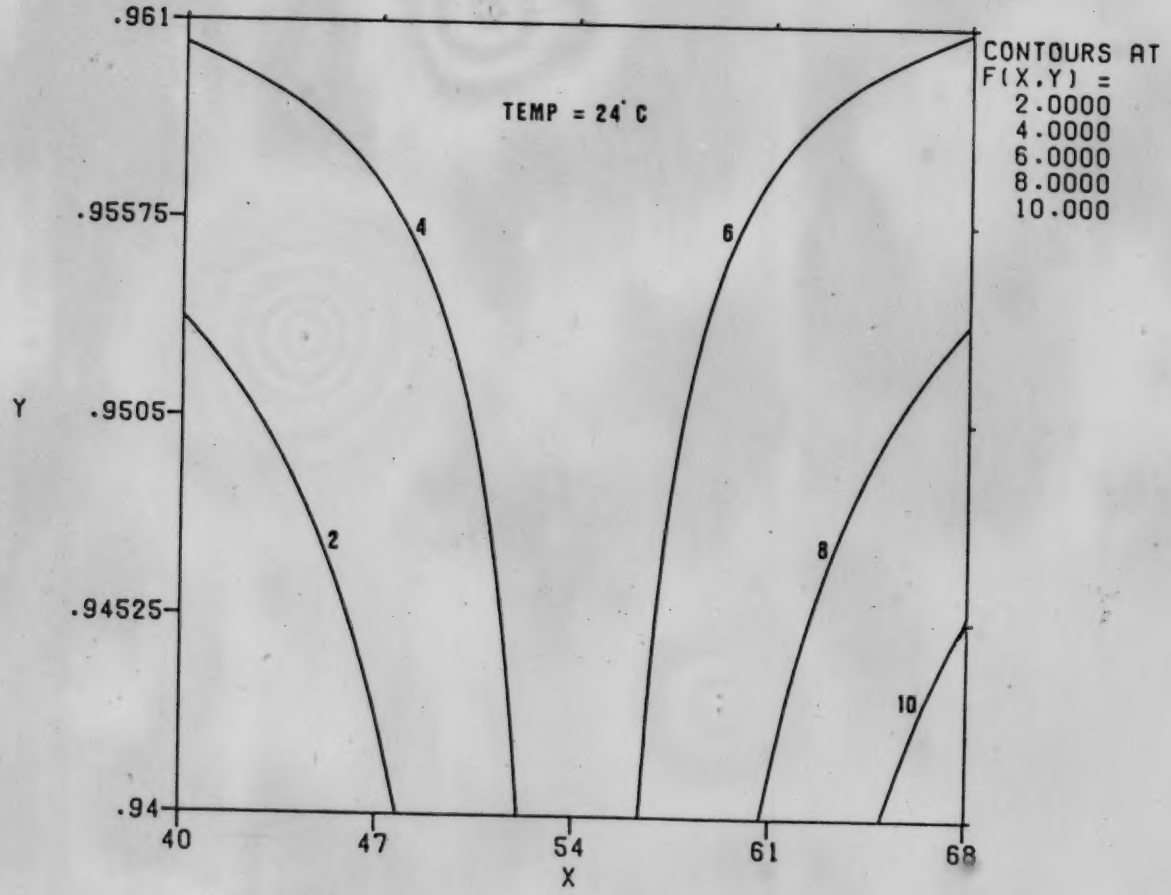


FIG. 4.18 Contour plot of days to visible mold growth for a_w (Y) versus CO_2 concentration (%)(X) with temperature held constant at 24°C.

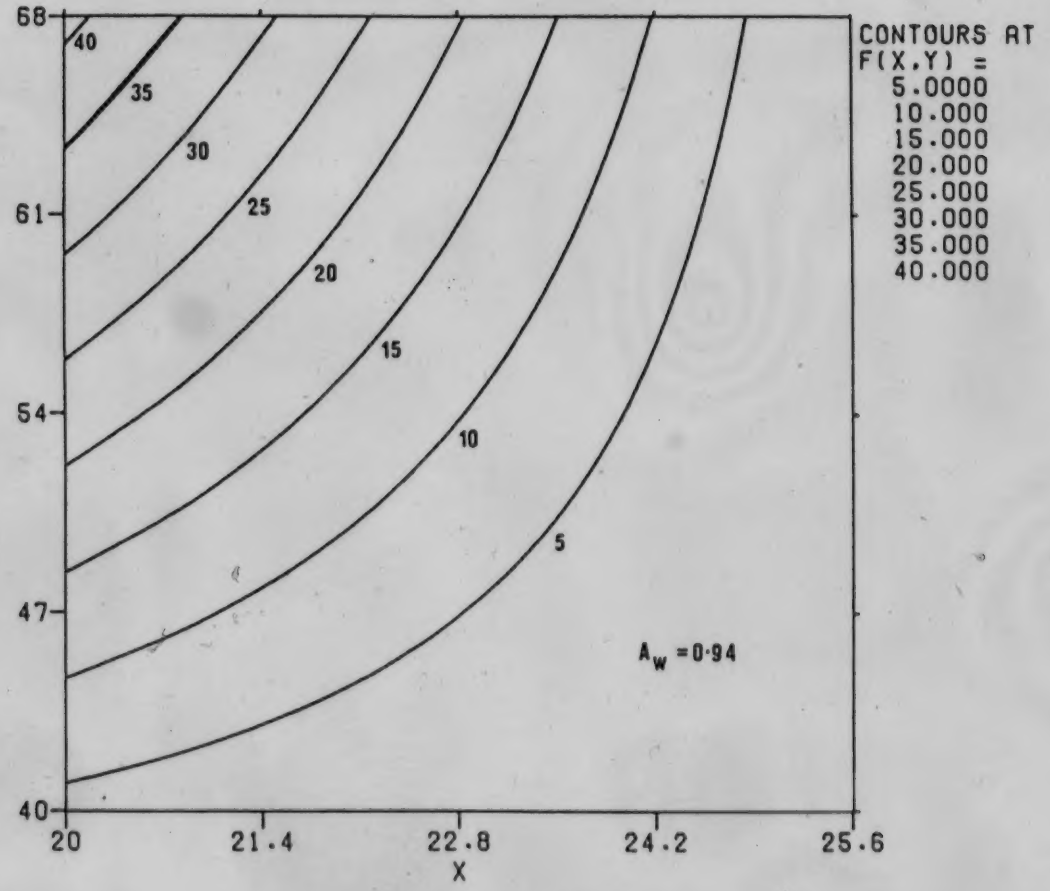


FIG. 4.19 Contour plot of days to visible mold growth for CO_2 (%) (Y) versus temperature (°C)(X) with a_w held constant at 0.94.

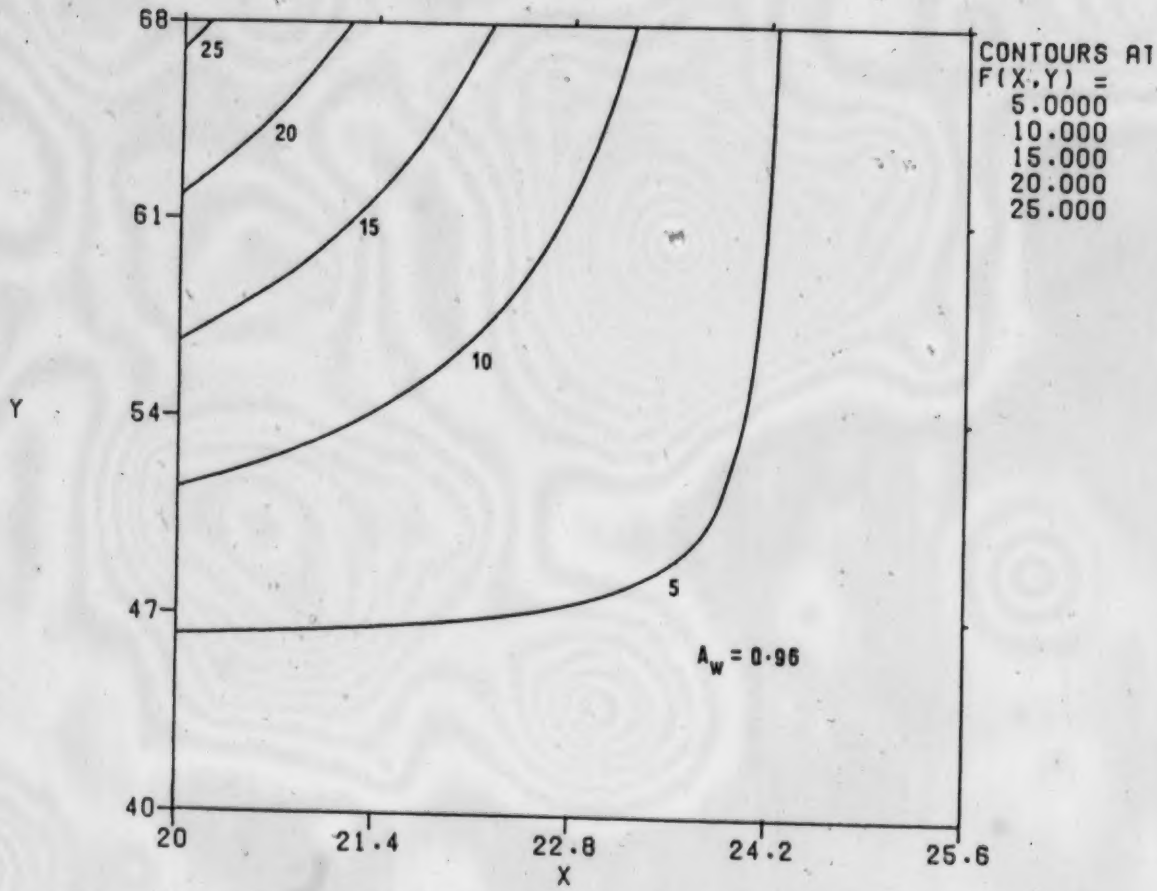


FIG. 4.20 Contour plot of days to visible mold growth for CO_2 (%) (Y) versus temperature (°C) (X) with a_w held constant at 0.96.

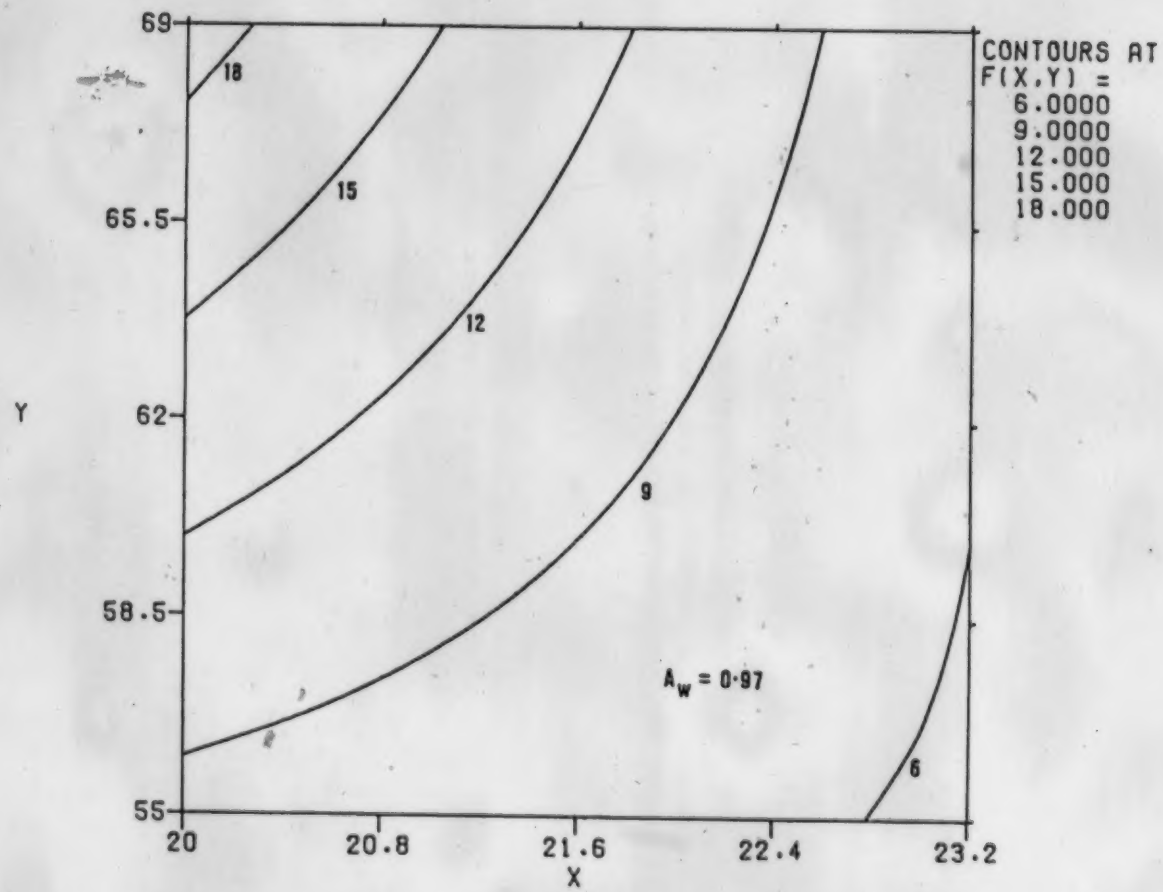


FIG. 4.21 Contour plot of days to visible mold growth for CO_2 (%) (Y) versus temperature (°C)(X) with a_w held constant at 0.97.

Perusal of these data confirms one important observation (Troller and Christian, 1980) that, as other environmental factors become more inhibitory, higher *aw* values are required for growth. Conversely, significant extensions of shelf life of high moisture bakery products could be obtained by increasing the concentration of CO_2 in the gas atmosphere. It is, however, evident from all contour plots that, in order to obtain a mold-free shelf life of 20-30 days in products with *aw* 0.95-0.96, temperature control is essential. This was adequately demonstrated in test bakes of crumpets made according to the recipe generated by the linear program (Table 5.5). The reformulated crumpets (*aw* 0.962) were packaged in 70% CO_2 and stored at 20°C and 30°C, respectively. Mold growth was evident in crumpets stored at 30°C after 7 days, whereas the gas packaged crumpets stored at 20°C remained mold-free even after 30 days. The experimental results obtained agree well with the predicted days to mold shown in Figs. 4.13-4.21. This again emphasizes the need for temperature control at all stages of distribution and storage in the retail environment in order to maximize the effect of other environmental factors used to inhibit mold growth.

Finally, the recommendation can be made to the manufacturer that packaging of crumpets in CO_2:Air mixtures is adequate for control of mold growth and the current N_2 content can be eliminated, with associated cost savings.

5. APPLICATION OF LINEAR PROGRAMMING TECHNIQUES TO REFORMULATION STUDIES OF CRUMPETS

Investigations revealed that the shelf life of gas packaged crumpets could be extended by a reduction in *aw* of the product from 0.98 to 0.96. This relatively small reduction in *aw* would be sufficient to inhibit the growth and heterofermentative activity of the predominant spoilage organism *Leuconostoc mesenteroides*. A method of reducing *aw* is the addition of expensive humectants such as glycerol and sorbitol. A viable alternative is reformulation of the Cane recipe to produce a final product with the desired level of free available water.

The traditional approach in reformulation studies has been a trial and error approach involving several test bakes until the desired proportions of raw ingredients were found. However a more economical and less laborious approach is the use of linear programming techniques.

5.1 Linear Programming Techniques

Linear programming was defined by Nicklin (1979) as "a mathematical technique used to determine the optimum allocation of a limited supply of resources, subject to certain constraints, to either maximize or minimize a specified objective". In reformulation studies of crumpets the resources were the raw ingredients, the constraints were based on chemical composition of the final product and the

objectives were cost minimization and reduction of a_w to a desired level.

According to Nicklin (1979), the advantages of this method over the traditional methods of reformulation are:

(a) removal of the guesswork and subjectivity normally associated with formulation studies,

(b) reduction of the time required for formulation work,

(c) cost minimization,

(d) rapid revision of formulation when required.

Linear programming techniques were therefore used in this study to:

(a) determine the levels of raw materials required to produce a final product within an a_w range of 0.965 - 0.955,

(b) minimize the cost of producing crumpets within these specifications.

The Linear Programming matrix is shown in Table 5.1.

The programs used were based on the conversational linear programming package of Smillie (1969).

5.2 Batch and Compositional Constraints

Constraints shown in the linear programming matrix (Table 5.1) were set on batch size of, and moisture, carbohydrate, protein and fat contents in, the final product. The ranges of the constraints for moisture, carbohydrate, protein and fat were based on the compositional analysis of the product as presently produced

TABLE 5.1 Linear Programming Matrix.

Ingredients	Cake flour (x_1)	5 Roses flour (x_2)	Whole wheat flour (x_3)	Salt (x_4)	Sodium bicarbonate (x_5)	Sodium aluminum phosphate (B_{260}) (x_6)	Monocalcium phosphate (V_{90}) (x_7)	Potassium sorbate (x_8)	Vinegar (x_9)	Water (x_{10})	
Cost/kg ($)	0.43	0.46	0.42	1.37	0.81	3.17	2.53	8.1	0.5	0.07	
Subject to Constraints											
Batch	x_1 +	x_2 +	x_3 +	x_4 +	x_5 +	x_6 +	x_7 +	x_8 +	x_9 +	x_{10} =	100 kg
Moisture	0.14	0.14	0.14	-	-	-	-	-	-	1	>48% <52%
Carbohydrate (as % starch)	0.85	0.79	0.71	-	-	-	-	-	-	-	>37% <43%
Protein	0.1	0.13	0.17	-	-	-	-	-	-	-	>5% <7%
Fat	0.01	0.01	0.016	-	-	-	-	-	-	-	>0.4% <0.6%
a_w in terms of g sucrose/g H_2O	0.2	0.2	0.2	11	3	4.6	4.2	1.9	0.8	-	>0.52 <0.73
Upper limits	x_1 =	x_2	3	<2	0.36	0.21	0.09	0.065	0.82	-	

(Table 5.2). Upper limits for bicarbonate, $B_{1.0}$, $V_{1.0}$, potassium sorbate and vinegar were based on the weights of these ingredients used by Forcrest Foods Ltd., Calgary, to produce a satisfactory product. Further limits were imposed by the Forcrest Foods' formulation. The ratio of cake flour to 5 Roses flour was maintained at 1:1 and whole wheat flour was limited to 3% of the final product. While salt was primarily used for organoleptic purposes, it can also be used as an inexpensive humectant to reduce *aw*. An upper limit of 2% was imposed on this ingredient as levels beyond this concentration produced an unacceptably salty product.

5.3 *aw* Constraints

The *aw* constraints set (0.97-0.955) were based on experimental values which were found to inhibit or delay the growth of, and gas production by, *Leuconostoc mesenteroides*. These were converted to sucrose concentration values (g sucrose/g water) by reference to a standard curve (Fig. 5.1), constructed from the experimental values provided by Seiler (1979) so that the contribution of all ingredients to the final *aw* could be incorporated using the following equation:

$$\text{Concentration of sucrose in aqueous phase of product (equivalent to } a_w\text{)} = \frac{aX_1 + bX_2 + cX_3 \ldots\ldots jX_{10}}{M_1 - M_2} \quad \text{——— 1}$$

TABLE 5.2 Proximate compositional analysis of English-style crumpets (Alberta Agriculture Food Laboratory, Edmonton).

COMPONENT	%
Moisture	48 - 52
Carbohydrate (as starch)	37 - 43
Protein	5 - 7
Lipid	0.4 - 0.6
Ash	1.4 - 1.8

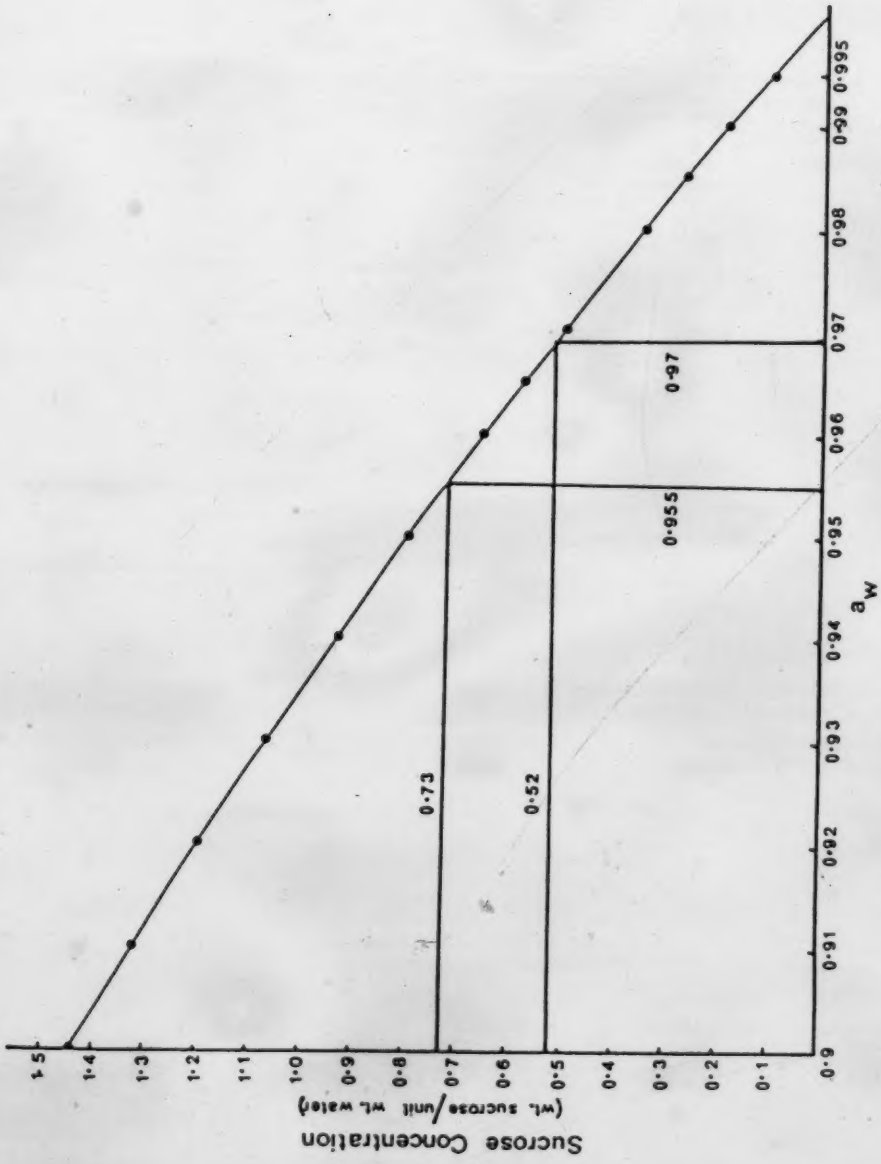


FIG. 5.1 a_w values of aqueous sucrose solutions.

where a,b,c,------i = Sucrose equivalents of ingredients

X_1, X_2, X_3---- X_{10} = Weights of ingredients

M_1 = Weight of water in ingredients

M_2 = Weight of water lost during baking and cooling

Note that the contribution of each ingredient to the final sucrose concentration in the aqueous phase is expressed as sucrose equivalents, i.e. g sucrose/g water which would give the same a_w as 1 g of material/g water. The sucrose equivalents (SE) of the ingredients used in the Forcrest Foods' formulation are shown in Table 5.3. For flour, salt and baking soda the sucrose equivalents were obtained from literature values (Seiler, 1979), whereas those for $B_{1.0}$, $V_{1.0}$, potassium sorbate and vinegar were determined as outlined in Section 5.5.1. Incorporation of these sucrose equivalents into Equation 1 for a_w values of ≤0.97 and ≥0.955, expressed in terms of sucrose concentration, is shown below:

$$\frac{0.2X_1 + 0.2X_2 + 0.2X_3 + 11X_4 + 3X_5 + 4.6X_6 + 4.2X_7 + 1.9X_8 + 0.8X_9}{M_1 - M_2} > 0.[illegible] \text{ g sucrose/g water} \quad \text{——— 2}$$

$$< 0.73 \text{ g sucrose/g water} \quad \text{——— 3}$$

Since flour and water were the major contributors to the moisture content (Mi) and subject to a 10% loss in weight, the denominator M_1-M_2 was limited to these ingredients to give:

$$\underset{(M_1)}{[0.14(X_1 + X_2 + X_3) + X_{10}]} - \underset{(M_2)}{0.1[0.14X_1 + 0.14X_2 + 0.14X_3) + X_{10}]} \quad \text{——— 4}$$

$$= 0.126X_1 + 0.126X_2 + 0.126X_3 + 0.9X_{10}$$

TABLE 5.3 Sucrose equivalents of some common bakery ingredients.

* Seiler, 1979.

INGREDIENT	SE
Flour	0.2 *
Salt	11 *
Baking soda	3 *
Sodium aluminium phosphate (B_{260})	4.6
Monocalcium phosphate (V_{90})	4.2
Potassium sorbate	1.87
Vinegar	0.81

The final equations used in the linear program were obtained by substituting Equation 4 into Equations 2 and 3:

$$0.2X_1 + 0.2X_2 + 0.2X_3 + 11X_4 + 3X_5 + 4.6X_6 + 4.2X_7 + 1.9X_8 + 0.8X_9 - 0.07X_1 - 0.07X_2 - 0.07X_3 - 0.47X_{10} > 0 \quad \text{——— 5}$$

$$0.2X_1 + 0.2X_2 + 0.2X_3 + 11X_4 + 3X_5 + 4.6X_6 + 4.2X_7 + 1.9X_8 + 0.8X_9 - 0.09X_1 - 0.09X_2 - 0.66X_{10} < 0 \quad \text{——— 6}$$

5.4 Further Compositional Data

The percent carbohydrate, fat, protein and moisture contents, respectively, of each ingredient were obtained by compositional analysis of the ingredients by the Alberta Agriculture Food Laboratory, Edmonton and are shown in Table 5.4. All values for carbohydrate, fat, protein and moisture were entered into the linear programming matrix (Table 5.1) in decimal form.

5.4.1 Costs of Ingredients

The costs of ingredients were entered in the linear programming matrix (Table 5.1) as $/kg.

5.5 Materials and Methods

5.5.1 Determination of Sucrose Equivalents

Sucrose equivalents were determined by the method recommended by Seiler (1979). A 2.5 g quantity of material was added to 20 g of a 60% (w/w) sucrose solution (equivalent to 1.5 g sucrose/g water). The mixture was

Table 5.4 Compositional analyses of ingredients used in crumpet formulation

Ingredient	% Composition			
	Moisture	Carbohydrate (as starch)	Protein	Lipid
Cake flour	14	84.8	10.3	1.01
5 Roses flour	14	79.3	12.9	0.94
Whole wheat flour	14	71.3	17.1	1.57
Salt	-	-	-	-
Sodium Bicarbonate	36.09	-	-	-
B_{260}	4.28	-	-	-
V_{90}	-	-	-	-
Potassium sorbate	0.04	-	-	-
50 Grain vinegar	89.35	-	-	-
Water	100	-	-	-

(Alberta Agriculture Food Laboratory, Edmonton)

heated at 100°C for 10 min, cooled and the *aw* measured using the manometric technique described in 3.2.3. The sucrose concentration (g sucrose/g water) equivalent to this *aw* was determined by reference to Fig. 5.1. Subtraction of the initially added sucrose (1.5 g/g water) from the reading gave the contribution of the experimental material (S), again in g sucrose/g water, to the *aw* measured.

The sucrose equivalent of the material was determined from the following formula:

$$\text{Sucrose equivalent of material} = \frac{S}{W}$$

where S = Contribution of material to *aw* reduction (g sucrose/g water)

W = g material/g water.

5.5.2 Test Bakes of Reformulated Product

Crumpets were made according to the formula generated by the linear program (Table 5.5) and baked on a Garland Griddle Model No. E22-186 (Russell Food Equipment Ltd., Edmonton, Alta) for 5 minutes at 350°F. The product was then cooled to room temperature before measurement of *aw* as outlined in Section 5.5.1.

5.6 Results and Discussion

The least cost formulation (weight of ingredients in the wet mix to give a final product weight of 100 kg) is shown in Table 5.5. The cost of ingredients for this formulation is 31 cents/kg, i.e. approximately 1.55 cents per crumpet. This cost compares favourably with the projected cost for 1982 of 1.51 cents/crumpet produced by Forcrest Foods Ltd., Calgary.

Good agreement was found between the actual a_w (0.962) and the predicted a_w (0.958) calculated as follows:

$$\text{Sucrose concentration (g sucrose/g water)} = \frac{0.2(24.78) + 0.2(24.78) + 0.2(3.04) + 11(1.962) + 3(0.36) + 4.6(0.21) + 4.2(0.09) + 0.8(0.82)}{0.126\,(X_1 + X_2 + X_3) + 0.9X_{10}}$$

$$\frac{35.03}{(0.126\,(24.78 + 24.78 + 3.04) + 0.9\,(49.72)}$$

$$\frac{35.03}{6.62 + 44.74} = \frac{35.03}{51.36} = 0.68 \text{ g sucrose/g water}$$

$$\equiv a_w \text{ of } 0.958$$

The correlation between the actual and theoretical a_w values indicates the accuracy of the compositional values and sucrose equivalents assigned to the various ingredients used in the linear program and the precision which can be obtained using a linear program under these conditions.

TABLE 5.5 Least cost formulation for crumpets.

INGREDIENT	WET WEIGHT (kg)
Cake Flour	24.78
5 Roses flour	24.78
Whole wheat flour	3
Salt	1.962
Sodium bicarbonate	0.36
Sodium aluminium phosphate (B_{262})	0.21
Monocalcium phosphate (V_{90})	0.09
Vinegar	0.82
Water	49.72
	105.76

Least cost analysis: $31.05

6. CONCLUSIONS

The objectives of this study have been met in that spoilage patterns of gas packaged crumpets, as presently produced, have been determined and the public health hazard, with regard to possible outgrowth of *Clostridium botulinum*, has been assessed. These studies have therefore provided the manufacturer with valuable information to allow him to plan, with greater confidence, his future production and marketing schedules, while alleviating immediate concerns on the safety of the product.

Such studies are, however, insufficient to adequately determine levels of environmental factors necessary to optimise shelf life and, therefore, increase market area and, ultimately, sales volume. The traditional experimental approach in shelf life studies is "one variable at a time". The major disadvantage of such experimental designs is that they examine the effect of each factor independently and, as a consequence, may under- or overestimate the level of each factor necessary to inhibit microbial growth. Futhermore, examination of several factors using this technique is laborious, time consuming and results in large quantities of data which may be rather difficult to interpret.

The unique feature of this study has been the use of Response Surface Methodology (RSM) to meet the important objective of increasing shelf life, which had been limited mainly by the growth and heterofermentative activity of *Leuconostoc mesenteroides*. Model studies on this spoilage

isolate demonstrated the value of RSM in quantifying the levels of environmental factors required to completely inhibit or suppress CO_2 production by this organism. For example, combinations of *aw* 0.986 and pH 5 could be used to completely inhibit growth at 20°C, while pH 5.6 would significantly reduce CO_2 production at the same *aw* and storage temperature (Fig. 3.10). Also, 22.5°C (Fig. 3.11), *aw* 0.975 and pH 5-5.4 could be employed to increase shelf life, while at 25°C (Fig. 3.12) reduction of *aw* to 0.96 and pH to 5-5.5 would be sufficient to delay CO_2 production. Thus, if spoilage problems were limited to the growth and metabolism of *Leuconostoc mesenteroides*, shelf life could be extended by judicious manipulation of *aw* and pH and packaging the reformulated product in air.

However, under such aerobic storage conditions shelf life would now be limited by mold growth. Initial screening studies indicated that CO_2, *aw* and storage temperature could be used to extend the mold free shelf life of the product. An RSM approach was again used to determine the levels of these factors necessary for optimisation of shelf life and possibly, since compressed air was used as the filler gas, for control of the outgrowth of *Clostridium botulinum*. Combinations of 64% CO_2, *aw* 0.94 and storage temperature of 20.4°C gave a predicted mold free shelf life of 32 days.

At slightly higher *aw*'s (0.96-0.97), which would be sufficient to inhibit or delay the growth of *Leuconostoc mesenteroides*, a mold free shelf life of 20-25 days could be

expected if the reformulated product was packaged in 70% CO_2 and stored at 20-21°C (Fig. 3.15).

It is obvious that, by using RSM, the manufacturer has at his disposal a much wider range of combinations of factors to control microbial growth than would be possible using the one variable at a time approach. Furthermore, it would be assumed that the data generated in this study could be applied to prevent similar spoilage problems in other gas packaged bakery products. Shelf life extension studies are presently being carried out on gas packaged waffles to test this hypothesis.

Thus, the advantages of RSM, which have been consistently demonstrated in food formulation studies and have now been further demonstrated in this study, are, according to Henika (1982):

(1) it provides more information in less time at less cost than traditional methods;

(2) it optimises combinations of ingredients and processing and storage conditions; and

(3) it presents results in an easy to understand graphical form.

While RSM has many advantages, it does have certain limitations. First of all, it is necessary to carefully select the level of environmental factors in each design to give a response. This was adequately demonstrated in the initial 2^{4-1} screening design in both the *Aspergillus niger* and *Leuconostoc mesenteroides* studies. Secondly, it should

be remembered that the results obtained by regression analyses are only estimates of shelf life for a particular set of data and may vary for another set of experimental data. And, thirdly, while the regression data can be used to predict extended shelf life, it is precarious to extrapolate too far outside the range of levels of factors actually used in experimental runs.

In this study the potential public health hazard of *Clostridium botulinum* appeared minimal in the presently produced crumpets (*aw* 0.98) packaged in CO_2:Air and CO_2:N_2 (70:30) gas atmospheres. The inability of the indigenous population of putrefactive anaerobes (*Clostridium sporogenes*) to reach levels $>10^3$ MPN/g was attributed to the low levels of spores initially present and the competitive edge of LAB. Results of the RSM study on *Leuconostoc mesenteroides* indicated that an *aw* of 0.96 would be sufficient to inhibit the growth of this organism and possible all other LAB present in the product. Further studies therefore need to be done using RSM to determine the levels of factors needed to ensure the public health safety of the reformulated gas packaged product in the absence of competing LAB. Indeed, it could be used to evaluate the whole area of microbiological standards and associated methodology.

Genigeorgis *et al.* (1971) used RSM to determine combinations of NaCl and pH to give a 2-6 log decrease in the number of *Staphylococcus aureus* cells in laboratory

media. While 6% NaCl (w/v) was required to give a 6 log decrease at pH 4, 14% NaCl was required to give the same reduction at pH 6. For a 2 log decrease in numbers, either 4% NaCl at pH 5.5 or 8% NaCl at pH 7 was needed to give the required reduction of cells. Such information is not only valid from a manufacturer's viewpoint, but also has an important impact on the development of realistic standards for *Staphylococcus aureus* in reformulated foods.

Extensive data have been compiled, using RSM, on the optimum concentration of ingredients required to make a wide range of food products. To date, no similar extensive studies have been conducted to determine the levels of environmental factors required to control the growth of the common food poisoning and food spoilage microorganisms. Such information would be of immense value in product development. All available data could be used to formulate products which would not only have superior sensory qualities but which would have an optimum shelf life and be microbiologically safe.

Despite its many advantages, RSM has been used sparingly in food microbiology studies. Only two other microbiological studies identifying RSM as the research tool have, apparently, been documented (Genigeorgis *et al.*, 1971; Schroder and Busta, 1973). According to Mead and Pike (1975), the limited use of RSM in biological studies is due to the fact that researchers, in general, are unaware of the technique. A further reason may be that microbiological

laboratories lack access to computational facilities necessary to avoid tedious and time consuming manipulation of data.

The use of RSM in microbiology is, therefore, in its infancy. Hopefully, through scientific publications, more and more researchers will become aware of the technique and of the obvious advantages it possesses over the classical approaches in applied food microbiological studies.

7. APPENDIX I

7.1 (a) Culture Media

7.1.1 (i) Modified Peptone Colloid Media (PCM; Greenburg *et al.*, 1966)

7.1.1.1 (a) PCM Broth

Bacto tryptose	20 g
Sodium chloride	5 g
Bacto agar	1 g
Dextrose	1 g
Ferrous sulphate $7H_2O$	0.365 g
Sodium thiosulphate	0.3 g
Distilled water	1 l

The ingredients were dissolved by boiling, dispersed in 9 ml amounts and sterilized by autoclaving at 121°C for 15 min. Final pH, 7.2.

7.1.1.2 (b) PCM Agar

As above, with the addition of 30 g/l of agar (Difco).

7.1.2 (ii) Tomato Glucose Milk Nutrient Agar (TGMNA) (Gibson and Abd-el-Malek, 1945)

Yeast extract	2.5 g
Dextrose	50 g
Tomato juice	100 ml

Reconstituted skim milk	800 ml
Nutrient agar	200 ml

The tomato juice and reconstituted skim milk were mixed together and the yeast and dextrose added. All ingredients were dissolved by boiling, dispensed in 9 ml amounts and sterilised by Tyndallisation (steaming for 30 min on each of 3 successive days). Final pH, 6.7.

7.2 (b) Reagents

7.2.1 (i) Mercuric chloride solution

Mercuric chloride	15 g
Concentrated hydrochloric acid	20 ml
Distilled water	100 ml

7.2.2 (ii) Vaspar seal

Petroleum jelly (melted)	500 ml
Mineral oil	500 ml

The mixture was air-sterilised in 250 ml amounts at 160°C for 2 hr.

8. APPENDIX II

8.1 Response Surface Methodology

Response surface methodology (RSM) is basically a group of multiple regression techniques applied to data generated by specified factorial designs. Such designs permit the factors of interest in experimental runs to be varied, not simply one at a time, but simultaneously.

Mathematical models, generally first and second order polynomials which describe the relationships between the response and input variables, are produced and ultimately manipulated to define the levels of the most significant factors required to optimize the response.

RSM consists of a number of sequential stages, which may include:

(a) *Screening designs.* Selection of the most significant factors which may influence response, using full or fractional factorial designs.

(b) *First-order designs.* Generation of a first-order polynomial model by multiple regression analysis of full factorial designs.

(c) *Path of steepest ascent.* Optimization of response within the limitations of the linear first-order model.

(d) *Second-order designs.* Extension of the mathematical description using a second-order polynomial model to include the possibilities of nonlinearity.

(e) *Canonical analysis.* Optimization of second-order response and full description of the response surface.

8.1.1 Screening Designs

Screening experiments are designed to determine a number of input variables, the most important variables which influence the response. When these are determined, they can be used in subsequent designs to optimize response.

Various designs can be employed in screening experiments. These include:

(a) Full factorial designs;

(b) Half fraction factorial designs; and

(c) Higher fraction factorial designs.

8.1.1.1 Full Factorial Designs

The effect of individual factors and their interactions can be evaluated by testing each factor in a two level factorial experiment. Such a design is often referred to as a 2^k design, where:

k = number of factors under investigation

2 = number of levels of each factor

A complete 2^k design for k=4 is shown in Table A1. The k-levels for each factor are referred to as "high" and "low" and define the operating range of interest for each factor in an experiment. The high and low levels of each factor are expressed by the presence or absence of lower case letters, respectively. Therefore, in run #2 factor A is at a high

TABLE A1 A complete 2^4 factorial design.

	LEVELS			
TC*	A	B	C	D
(-1)	-1	-1	-1	-1
(a)	1	-1	-1	-1
(b)	-1	1	-1	-1
(ab)	1	1	-1	-1
(c)	-1	-1	1	-1
(ac)	1	-1	1	-1
(bc)	-1	1	1	-1
(abc)	1	1	1	-1
(d)	-1	-1	-1	1
(ad)	1	-1	-1	1
(bd)	-1	1	-1	1
(abd)	1	1	-1	1
(cd)	-1	-1	1	1
(acd)	1	-1	1	1
(bcd)	-1	1	1	1
(abcd)	1	1	1	1

*Treatment combination.

level and factors B, C and D are at low levels; whereas in run #18 all factors are investigated at the high level.

It is often more convenient to "code" the independent variables with +1 (high level) and -1 (low level). The method of coding the independent variables is outlined in Table A2. For example, if we have 2 temperatures under study, namely 20 and 30°C, the coded variable for 20°C would be, on the basis of the above equation:

$$\frac{20°-\frac{1}{2}(20°+30°)}{\frac{1}{2}(30°-20°)}$$

$$= \frac{20-25}{5} = -1$$

A temperature of 30°C would, therefore, be coded as +1.

The results of the full factorial design are analysed by standard regression techniques, resulting in a fitted linear mathematical model, an example of which is shown in Fig. A1. The significance of each estimated coefficient is tested by a Student's two-tailed t-test. Nonsignificant main effects and interactions at the 0.05 level are normally subsequently discarded.

A 2^4 factorial design, therefore, estimates the coefficients associated with the 4 main-factor, 6 two-factor, 4 three-factor and 1 four-factor interaction effects. In terms of their importance, higher order interactions (3 or more factors) are assumed to have a negligible effect on a response. According to Box *et al.*

TABLE A2 General equation used to code input variables.

$$\text{CODED VARIABLE} = \frac{\text{ACTUAL VALUE} - 1/2\ (\text{HIGH VALUE} + \text{LOW VALUE})^{*}}{1/2\ (\text{HIGH VALUE} - \text{LOW VALUE})^{\dagger}}$$

*NUMERATOR = BASE LEVEL

†DENOMINATOR = UNIT

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D$$

$$+ \beta_{1,2} AB + \beta_{1,3} AC + \beta_{1,4} AD + \beta_{2,3} BC + \beta_{2,4} BD + \beta_{3,4} CD$$

$$+ \beta_{1,2,3} ABC + \beta_{1,2,4} ABD + \beta_{1,3,4} ACD + \beta_{2,3,4} BCD$$

$$+ \beta_{1,2,3,4} ABCD + \epsilon$$

FIG. A1 Linear regression model of a complete 2^4 factorial design.

(1978), a considerable reduction in the number of experimental runs can be made by giving up the "redundant information" in 2^k designs and using fractional factorial designs as screening designs.

8.1.1.2 Half Fraction Designs

A half fraction design, as the name imples, utilises only half of the possible runs, i.e. 2^{k-1} of the full factorial design (2^k). For example a half fraction design for 4 factors involves only 8 runs, with a consequent loss of accuracy of the estimated regression coefficients.

Selection of the 8 runs cannot be done at random, but must follow a specific procedure to ensure that equal levels of all main effects are obtained. For example, if the first 8 runs of the full 2^4 factorial design (Table A1) were chosen, there would be equal levels for factors A, B and C, but only 1 level of D and, therefore, no estimate of variation for D. To ensure that equal levels of each factor are included in the half fraction design, specific procedures have been developed (Box *et al.*, 1978). The first step is to select the "best defining relation", which is usually the highest order interaction term. For a 2^k design where k=4, the defining relation would be I=ABCD. The value of I is obtained by multiplying the levels of the A, B, C and D columns so that I=ABCD will be equal to +1 or -1, which define the experimental runs. In order to obtain the specific 2^{k-1} design, all runs with either I=+1 or -1 for

the defining relation are selected. The design chosen, i.e. I=ABCD (+1), is shown in Table A3. This design contains equal levels of each factor, i.e. 4 at the +1 level and 4 at the -1 level, and, therefore, enables variation due to these factors to be determined.

When the 8 runs of the I=ABCD fraction are written out in full, certain relationships between pairs of columns become evident; namely, A=BCD, B=ACD, C=ABD, D=ABC, AB=CD, AC=BD and AD=BC. These relationships are shown in Table A4. The effect of A is, therefore, "mixed up" or "confounded" with BCD; B with ACD, and so on. As a result of this confounding pattern, the parameter β_1 in the term $\beta_1 A$ in the linear model shown in Fig. A1 is actually estimated from $\beta_1 A + \beta_{234} BCD$. This is more conveniently expressed as $l_A = \beta_1 + \beta_{234}$, where l_A is the linear combination of y's that estimate β_1. Therefore, in a 2^{4-1} design the following estimates are obtained:

$$l_A = \beta_1 + \beta_{234} \quad (A + BCD)$$

$$l_B = \beta_2 + \beta_{134} \quad (B + ACD)$$

$$l_C = \beta_3 + \beta_{124} \quad (C + ABD)$$

$$l_D = \beta_4 + \beta_{123} \quad (D + ABC)$$

$$l_{AB} = \beta_{12} + \beta_{34} \quad (AB + CD)$$

$$l_{AC} = \beta_{13} + \beta_{24} \quad (AC + BD)$$

$$l_{AD} = \beta_{14} + \beta_{23} \quad (AD + BC)$$

$l_I = \frac{1}{8}(y_1+y_2+y_3+y_4+y_5+y_6+y_7+y_8)$, i.e. mean of 8 runs.

The estimated coefficients in a 2^{4-1} design are, therefore, less precise than the coefficients extimated in a full 2^k

TABLE A3 A 1/2 fraction of a 2^4 factorial design (I=ABCD)

TC*	A	B	C	D	ABCD
(1)	-1	-1	-1	-1	1
(ad)	1	-1	-1	1	1
(bd)	-1	1	-1	1	1
(ab)	1	1	-1	-1	1
(cd)	-1	-1	1	1	1
(ac)	1	-1	1	-1	1
(bc)	-1	1	1	-1	1
(abcd)	1	1	1	1	1

*Treatment combination

TABLE A4 Pattern of a 2^{4-1} design (I=ABCD).

TC*	I	A	B	C	D	AB	AC	AD	BC	BD	CD	ABC	ABD	ACD	BCD	ABCD
(1)	1	-1	-1	-1	-1	1	1	1	1	1	1	-1	-1	-1	-1	1
(ad)	1	1	-1	-1	1	-1	-1	1	1	-1	-1	1	-1	-1	1	1
(bd)	1	-1	1	-1	1	-1	1	-1	-1	1	-1	1	-1	1	-1	1
(ab)	1	1	1	-1	-1	1	-1	-1	-1	-1	1	-1	-1	1	1	1
(cd)	1	-1	-1	1	1	1	-1	-1	-1	-1	1	1	1	-1	-1	1
(ac)	1	1	-1	1	-1	-1	1	-1	-1	1	-1	-1	1	-1	1	1
(bc)	1	-1	1	1	-1	-1	-1	1	1	-1	-1	-1	1	1	-1	1
(abcd)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

* Treatment combination.

design. However, this loss of precision is not critical as the linear coefficients of β_1, β_2, β_3 and β_4 are confounded with higher order interactions which are not expected to make a significant contribution to the linear model.

8.1.1.3 Higher Fraction Factorial Designs

Higher fraction factorial designs (2_k^{-p}, where p>1) are used when k is large and it is no longer practical to perform even a half fraction factorial design. An example of such a design and the one used in this study is a 2^{6-3} design with generating relations I=ABD, I=ACE and I=ABCF (Box *et al.*, 1978). The design, involving only 8 experimental runs, is shown in Table A5. According to Box *et al.* (1978), when a design has more than one generating relation the defining relation must include each generator and all possible combinations of these generators. The defining relation for this particular 2^{6-3} design is shown in Table A6. The confounding pattern, shown in Table A7, is determined by multiplying the final defining relation, I, on both sides by the factors of interest, which in our case are the main factors. By neglecting 3 factor or higher interaction effects, the linear combinatons of y's, which estimate the linear regression coefficients, are obtained (Table A8). Again, using a Student's two-tailed t-test, the significance of each factor is determined.

TABLE A5 A 2^{6-3} factorial design with generating relations I = ABD, I = ACE, I = ABCF.

TC*	A	B	C	D	E	F	I
1	-1	-1	-1	1	1	-1	1
2	1	-1	-1	-1	-1	1	1
3	-1	1	-1	-1	1	1	1
4	1	1	-1	1	-1	-1	1
5	-1	-1	1	1	-1	1	1
6	1	-1	1	-1	1	-1	1
7	-1	1	1	-1	-1	-1	1
8	1	1	1	1	1	1	1

* Treatment combination.

TABLE A6 Defining relation (I) for a 2^{6-3} factorial design with generating relations I = ABD, I =ACE, I = ABCF.

I = ABD = ACE = ABCF	(GENERATORS ONE AT A TIME)
= BCDE = CDF = BEF	(MULTIPLYING GENERATORS 2 AT A TIME)
= ADEF	(MULTIPLYING GENERATORS 3 AT A TIME)

TABLE A7 Confounding pattern of a 2^{6-3} factorial design

MAIN FACTOR	ALIASES
A	= BD = CE = BCF = ABCDE = ACDF = ABEF = DEF
B	= AD = ABCE = ACF = CDE = BCDF = EF = ABDEF
C	= ABCD = AE = ABF = BDE = DF = BCEF = ACDEF
D	= AB = ACDE = ABCDF = BCE = CF = BDEF = AEF
E	= ABDE = AC = ABCEF = BCD = CDEF = BF = ADF
F	= ABDF = ACEF = ABC = BCDEF = CD = BE = ADE

TABLE A8 Combination of effects used to estimate linear regression coefficients of a first order model.

ℓ_A = A + BD + CE $(\beta_1 + \beta_{2,4} + \beta_{3,5})$

ℓ_B = B + AD + EF $(\beta_2 + \beta_{1,4} + \beta_{5,6})$

ℓ_C = C + AE + DF $(\beta_3 + \beta_{1,5} + \beta_{4,6})$

ℓ_D = D + AB + CF $(\beta_4 + \beta_{1,2} + \beta_{3,6})$

ℓ_E = E + AC + EF $(\beta_2 + \beta_{1,3} + \beta_{5,6})$

ℓ_F = F + CD + BE $(\beta_6 + \beta_{3,4} + \beta_{2,5})$

ℓ_I = MEAN OF THE 8 RUNS (β_o)

8.1.1.4 First Order Polynomial Design

To this stage, screening designs have permitted selection of important variables from all those originally considered to possibly influence the response. A full factorial design, using only the factors considered important (k), is now performed. This is the basis of the first order polynomial design. High and low levels of each variable are again selected and coded to equal +1 or -1. Additional runs (n) are added at base levels (0), the linear mid-point between +1 and -1, to measure experimental error. There is no limitation on the number of additional runs other than laboratory resources.

The general formula for the first order polynomial design is 2^k+n, an example of which, for k=3 and n=4, is shown in Table A9. The results of this design are analysed by standard multiple regression techniques and enable the first order polynomial model (shown below) to be fitted, i.e.:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \varepsilon$$

where B_1, B_2 and B_3 are the regression coefficients associated with A, B and C (X_1, X_2, and X_3). The significance of each regression coefficient is again tested using the two-tailed t-test.

TABLE A9 First order polynomial design (2^k+n) for k = 3, n = 4.

	LEVELS		
TC*	A	B	C
1	-1	-1	-1
2	1	-1	-1
3	-1	1	-1
4	1	1	-1
5	-1	-1	1
6	1	-1	1
7	-1	1	1
8	1	1	1
9	0	0	0
10	0	0	0
11	0	0	0
12	0	0	0

*Treatment combination.

8.1.1.5 Path of Steepest Ascent

The path of steepest ascent is a method whereby the experimenter moves sequentially along a path towards a maximum response. The direction of the path of steepest ascent (or descent) is determined from the estimated regression coefficient of the first order linear equation. An example of the path of steepest ascent approach is shown in Table A10.

When an increase in response is continually indicated, a further first order design may be run using, as base levels, the levels which gave the most favorable response in the path of steepest ascent. When no further increase is indicated or estimated coefficients of the interaction terms in the linear model are greater than the linear coefficients, a stationary region has been reached. A more elaborate design is needed to explore the local surface at this stationary region.

8.1.1.6 Second Order Designs

The nature of the stationary region can be determined by a second order design. Fitting of the second order polynomial equation is shown below for 3 factors.

$$y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$

To estimate the regression coefficients (β) in this model, the experimental design must include at least 3 levels of each factor if estimates of curvature along each axis are to be made. A 3^k factorial design is the simplest but is only

TABLE A10 Path of steepest ascent approach for CO_2 production.

	A (a_w)	B (pH)	C (Temp °C)	
BASE LEVEL	0.98	6.25	25	
UNIT CHANGE	0.01	1	5	
ESTIMATED SLOPE (β)	2.190	2.513	1.325	
STEEPEST ASCENT VECTOR (UNIT CHANGE x SLOPE)	0.02	2.51	6.63	
STEEPEST ASCENT VECTOR (FOR 0.005 CHANGE IN a_w)	0.005	0.58	1.52	
				PREDICTED CO_2 (ml)
PATH OF STEEPEST ASCENT	0.955	3.4	17.4	(-5.5)
	0.96	3.95	18.9	(-2.6)
	0.965	4.52	20.45	(0.38)
	0.97	5.1	21.9	(3.32)
	0.975	5.68	23.5	(6.26)
	0.98	6.25	25	(9.2)
	0.985	6.8	26.5	(12.1)
	0.99	7.3	28	(15.1)
	0.995	7.9	29.5	(18)

satisfactory for a small number of factors (k=2 or 3).

To reduce the number of experimental runs for larger k values, central composite designs (CCD) (Box and Wilson, 1951) are normally used. These are simply 2^k factorial designs augmented by additional points to give $2^k + 2k + 1$ treatment combinations.

The construction of a CCD for k=3 and k=4 is outlined in Tables A11 and A12, respectively. The geometrical representation of a CCD for k=3 is shown in Fig. A2. The number of experimental runs required for a 3^k design and CCD for k=2, 3, 4 and 5 is shown in Table A13.

When the second order equation is expressed in matrix notation, the following equation is obtained:

$$Y = \begin{bmatrix} X_1 X_2 X_3 \end{bmatrix} \begin{bmatrix} \beta_1 \\ \beta_2 \\ \beta_3 \end{bmatrix} + \begin{bmatrix} X_1 X_2 X_3 \end{bmatrix} B \begin{bmatrix} X_1 \\ X_2 \\ X_3 \end{bmatrix}$$

which gives:

$$Y = X\beta + X^{I}BX$$

where:

X is a matrix of $X_1 X_2 X_3$

X' is the transposed matrix of $X_1 X_2 X_3$

β is a matrix of the linear coefficients

B is a matrix of coefficients associated with the quadratic and interaction terms in the model.

TABLE A11 Construction of a Central Composite Design ($2^k + 2k + 1$) where k = 3.

LEVELS		
A	B	C
-1	-1	-1
1	-1	-1
-1	1	-1
1	1	-1
-1	-1	1
1	-1	1
-1	1	1
1	1	1
$-\alpha$	0	0
α	0	0
0	$-\alpha$	0
0	α	0
0	0	$-\alpha$
0	0	α
0	0	0

TABLE A12 Construction of a Central Composite Design ($2^k + 2k = 1$) where k = 4.

LEVELS

A	B	C	D
-1	-1	-1	-1
1	-1	-1	-1
-1	1	-1	-1
1	1	-1	-1
-1	-1	1	-1
1	-1	1	-1
-1	1	1	-1
1	1	1	-1
-1	-1	-1	1
1	-1	-1	1
-1	1	-1	1
1	1	-1	1
-1	-1	1	1
1	-1	1	1
-1	1	1	1
1	1	1	1
α	0	0	0
$-\alpha$	0	0	0
0	α	0	0
0	$-\alpha$	0	0
0	0	α	0
0	0	$-\alpha$	0
0	0	0	α
0	0	0	$-\alpha$
0	0	0	0

TABLE A13 Experimental runs in a 3 level factorial design and a Central Composite Design for k = 2, 3, 4 and 5.

DESIGN	NO. OF EXPERIMENTAL RUNS			
3 LEVEL FACTORIAL (3^k)	9	27	81	245
CENTRAL COMPOSITE DESIGN ($2^k + 2k + 1$)	9	15	25	43

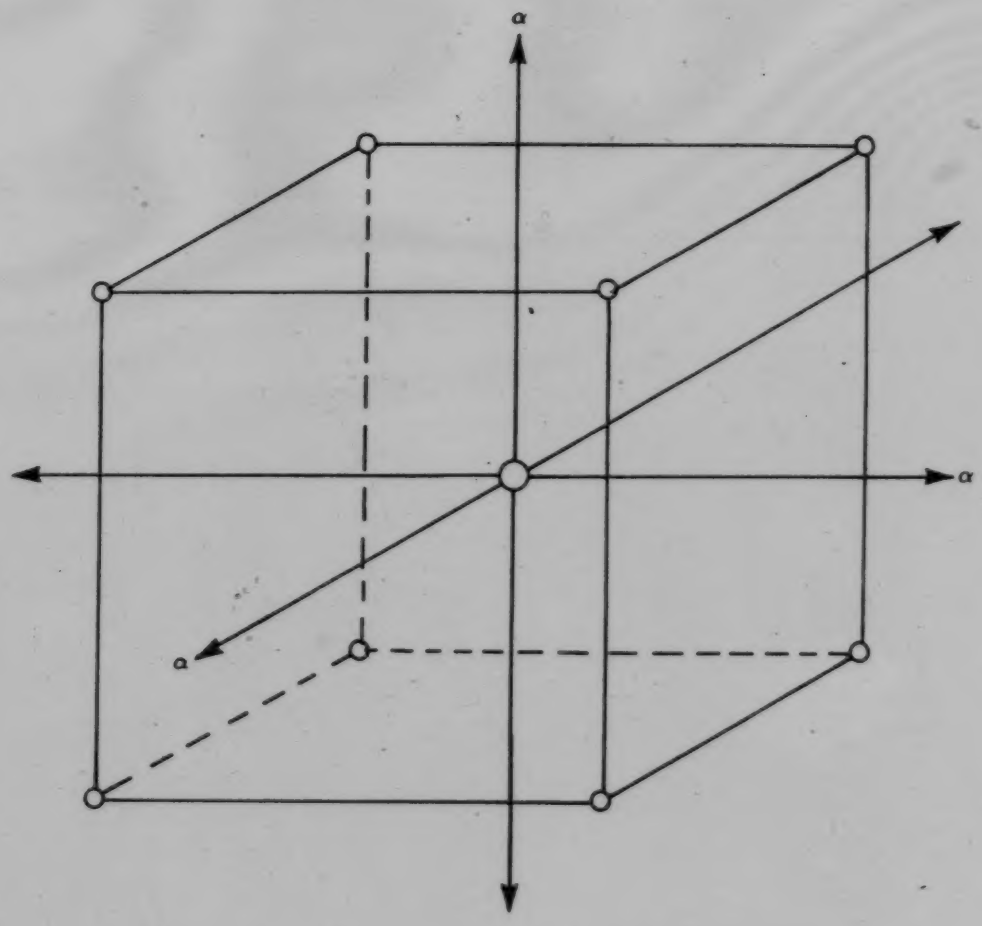


FIG. A2. Geometrical representation of a Control Composite Design for k = 3. (Myers, 1976)

When this equation is differentiated with respect to X, equated to zero and solved, a unique point, the stationary point (X_s), is found. The stationary point represents the point on the response surface where the slope is equal to zero, and is computed by the equation shown in Table A14.

The estimated yield at the stationary point can be calculated from the equation shown in Table A15.

8.1.1.7 Canonical Analysis

The value of X_s, computed previously from the second order equation, provides little information about the nature of the fitted surface. To determine the nature of the stationary point and the response surface, the second order equation is more conveniently expressed as a canonical equation (Myers, 1976), an example of which, for 3 variables, is shown below:

$$Y = Y_0 + \lambda w_1^2 + \lambda w_2^2 + \lambda w_3^2$$

where:

Y_s = estimated response at the stationary point (X_s)

λ = eigenvalues of the matrix B

w = canonical variables (linear combinations of the original variables X_1, X_2, X_3

A graphical illustration of the canonical form for a response surface in two variables is shown in Fig. A3.

TABLE A14 Calculation of the stationary points on the fitted surface for a Central Composite Design (k = 3)

$$X_0 = \frac{-B^{-1}.b}{2}$$

WHERE $B = \begin{bmatrix} \beta_{1,1} & \frac{\beta_{1,2}}{2} & \frac{\beta_{1,3}}{2} \\ \frac{\beta_{2,1}}{2} & \beta_{2,2} & \frac{\beta_{2,3}}{2} \\ \frac{\beta_{3,1}}{2} & \frac{\beta_{3,2}}{2} & \beta_{3,3} \end{bmatrix}$ I E. MATRIX OF REGRESSION COEFFICIENTS ASSOCIATED WITH CROSS-PRODUCTS

$b = \begin{bmatrix} \beta_1 \\ \beta_2 \\ \beta_3 \end{bmatrix}$ I.E. LINEAR REGRESSION COEFFICIENTS MATRIX

TABLE A15 Equation to estimate yield at the stationary point.

$$Y_0 = \beta_0 + \frac{X_0^1 . \beta}{2}$$

WHERE β_0 = THE INTERCEPT POINT FROM THE FITTED EQUATION

X_0^1 = TRANSPOSED MATRIX OF VALUES AT THE STATIONARY POINT

β = LINEAR REGRESSION COEFFICIENT MATRIX

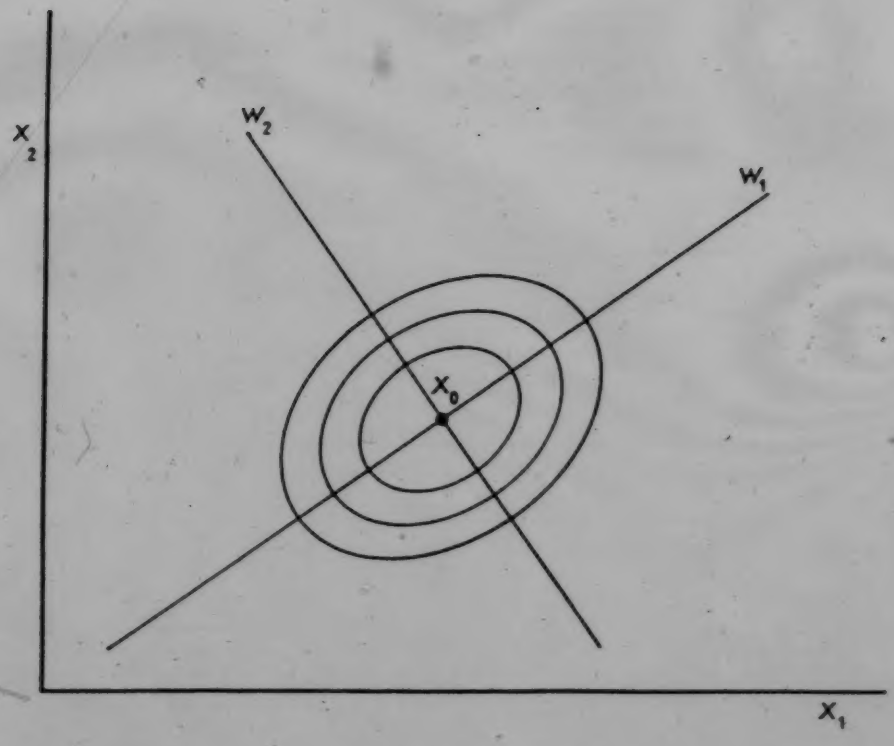
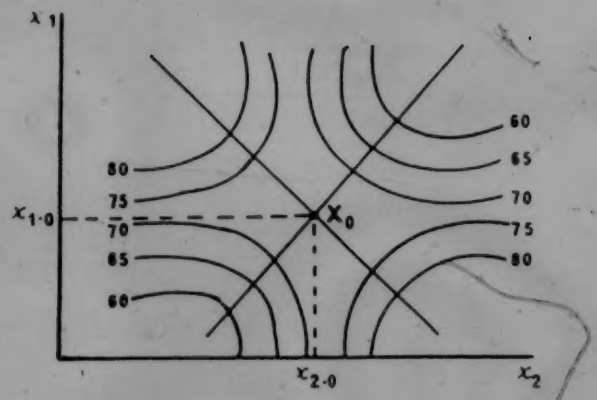


FIG. A3 Illustration of canonical form for a response surface in two variables (Myers, 1976).

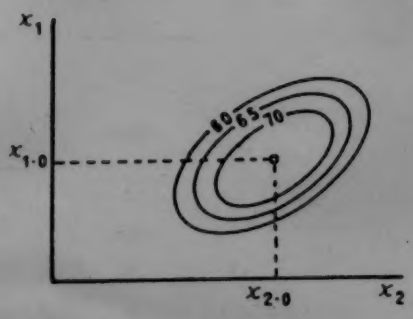
The signs and magnitude of the eigenvalues (λ) in the canonical equation provide valuable information about the nature of the fitted surface. For example, when the eigenvalues are all negative, X_s is a point of maximum response on the fitted surface and a move in any direction along any of the w-axis would result in a decrease in response. However, if the eigenvalues are all positive, X_s on the fitted surface has reached a point of minimum response and a move in any direction along the w-axis would increase the response.

A more common situation, and one found in this study, is both positive and negative values in the canonical equation. The interpretation of this situation is that the response Y_s at this particular X_s is neither a maximum or minimum response, and is commonly referred to as a "saddle point". A move along one direction on the w-axis would increase the response, while a move in another direction would give a decrease in response. Examples of a minimum, a maximum and a saddle point on the fitted surface are shown in Fig. A4.

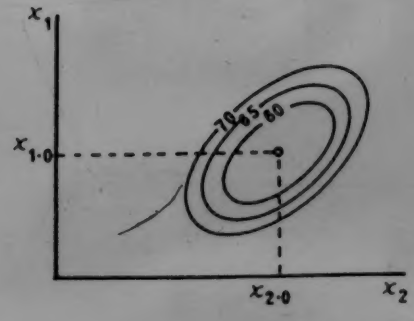
When a saddle point situation develops, the experimenter is interested in finding the levels of variables which would increase the response. These levels are found from the relationship $w = M'Z$ (Myers, 1976) an example of which, for three variables, is shown in Fig. A5. When selected values of w_1, w_2 and w_3 are used in this equation, values of X_1, X_2 and X_3, within the experimental



Saddle point in a fitted surface.



Maximum point in a fitted surface.



Minimum point in a fitted surface.

FIG. A4 Examples of a minimum, a maximum and a saddle point on the fitted surface (Myers, 1976).

$$w = M^{1} Z$$

WHERE w = CANONICAL VARIABLES

M^{1} = TRANSPOSED MATRIX OF EIGENVECTORS

$Z = (X - X_o)$

$$\begin{bmatrix} w_1 \\ w_2 \\ w_3 \end{bmatrix} = \begin{bmatrix} 0.0529 & -0.9416 & 0.3325 \\ -0.3325 & 0.2734 & 0.8950 \\ 0.9416 & 0.1579 & 0.2793 \end{bmatrix} \begin{bmatrix} x_1 - 1.24 \\ x_2 - 0.95 \\ x_3 + 0.655 \end{bmatrix}$$

FIG. A5 Procedure for generating transformed variables (Myers, 1976).

range, will be computed to assist in locating the optimum response on the fitted surface.

In summation, response surface methodology is an elegant statistical package which was used in this study to determine: (1) the significant environmental factors influencing the growth of selected spoilage isolates of crumpets. (2) the levels of these factors, within the constraints of the presently produced product, required to inhibit or delay microbial growth and activity and so optimize product shelf life.

For a more detailed account of RSM and the rationale of confounding, the reader should consult the texts of Myers (1976), Davies (1967) and Box *et al*. (1978).

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